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Eira Kelo

Catalytic and Therapeutic Characteristics of Human Recombinant Glycosylasparaginase and Bacterial L-asparaginases

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EIRA KELO

Catalytic and therapeutic characteristics of human recombinant glycosylasparaginase and bacterial L-asparaginases

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Catalytic and therapeutic characteristics of human recombinant glycosylasparaginase and bacterial L-asparaginases

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ABSTRACT

Glycosylasparaginase (GA) is a lysosomal enzyme which cleaves the linkage between N-acetylglucosamine and L-asparagine in asparagine-linked glycoproteins. The deficiency of GA in aspartylglucosaminuria (AGU) patients leads to progressive psychomotor retardation and a shortened life span due to the massive accumulation of aspartylglucosamine (GlcNAc-Asn) and other glycoasparagines into tissues and body fluids. Currently there is no curative therapy available for AGU. Development of treatment strategies is challenging due to the fact that AGU also affects the central nervous system (CNS).

In the first part of the present study, the effectiveness of enzyme replacement therapy (ERT) with human recombinant GA was evaluated in the mouse model of AGU by using biochemical methods. It was found that intravenously administered GA effectively cleared GlcNAc-Asn from the non-neuronal tissues of the treated adult AGU mice, and surprisingly, to some extent also from brains. Moreover, a glycoasparagine $Man_2GlcNAc_2$-Asn was present as high concentrations in the non-neuronal tissues of the untreated AGU mice, whereas its accumulation in brain was negligible. The level of $Man_2GlcNAc_2$-Asn in liver and spleen increased with age of the mice, but no such similar trend was found in other tissues. ERT with intraperitoneal injections of GA effectively removed both GlcNAc-Asn and $Man_2GlcNAc_2$-Asn from non-neuronal tissues of adult AGU mice.

In the second part of the study, the enzymatic properties of GA and asparaginase were compared. In addition to GlcNAc-Asn, GA is known to hydrolyze β-aspartylpeptides. Encouraged by the structural similarity of GA and bacterial asparaginases, it was possible to demonstrate that bacterial L-asparaginases, which are used in the treatment of acute lymphoblastic leukemia (ALL), are also able to catalyze the hydrolysis of certain β-aspartylpeptides, especially small size β-aspartyl amino acid amides *in vitro*. The results suggest that therapy with bacterial L-asparaginases may affect the degradation of β-aspartylpeptides in human body and may probably cause some of the side effects.

Due to the fact that GA has similar L-asparaginase activity as the bacterial L-asparaginases used in ALL therapy, it was hypothesized that GA might be able to induce apoptosis in leukemia cells *in vitro.* By hydrolyzing the L-asparagine reservoirs, GA induced cell death especially in B-lineage ALL cell culture, and less efficiently in T-lineage ALL cells, in a comparable manner with bacterial L-asparaginases. It was also found that human recombinant GA depleted effectively both intra- and extracellular reservoirs of L-asparagine in the EBV-transformed GA-deficient lymphoblast culture.

This study provides new insights into the enzymatic and therapeutic properties of glycosylasparaginase suggesting that since it is an endogenous human protein, it might be applicable as a potential anti-cancer agent as well as being used in the therapy of AGU.

National Library of Medicine Classification: QU 55, QU 135, WB 330, WD 105, WH 250

Medical Subject Headings: Enzyme Replacement Therapy; Enzymes/therapeutic use; Aspartylglucosylaminase; Asparaginase; Aspartylglucosaminuria; N-acetylglucosaminylasparagine; Recombinant Proteins; Disease Models, Animal; Mice; Leukemia; Cells, Cultured; Apoptosis

Kelo, Eira

Rekombinantin ihmisperäisen glykosyyliasparaginaasin sekä bakteeriperäisten L-asparaginaasien katalyyttiset ja terapeuttiset ominaisuudet

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TIIVISTELMÄ

Aspartyyliglukosaminuria (AGU) on suomalaiseen tautiperintöön kuuluva peittyvästi periytyvä lysosomaalinen kertymäsairaus, joka johtuu mutaatiosta glykosyyliasparaginaasi-geenissä. Glykosyyliasparaginaasi-entsyymin puuttuminen aiheuttaa aspartyyliglukosamiinin ja muiden sokerijohdannaisten kertymisen elimistön soluihin, mikä johtaa henkisen ja fyysisen suorituskyvyn heikkenemiseen ja lopulta vaikeaan kehitysvammaisuuteen ja ennenaikaiseen kuolemaan. Tällä hetkellä AGU-tautiin ei ole parannuskeinoa. Hoitomenetelmien suunnittelu ja toteutus on haasteellista, sillä AGU vaikuttaa myös keskushermostoon.

Väitöskirjan osatavoitteena oli selvittää entsyymikorvaushoidon tehokkuus AGU-hiirissä. Soluviljelmässä tuotetulla rekombinantilla glykosyyliasparaginaasi-entsyymillä hoidettujen aikuisten AGU-hiirten kudosten aspartyyliglukosamiinipitoisuus aleni merkittävästi varsinkin suurilla entsyymiannoksilla. Glykosyyliasparaginaasi-entsyymin aktiivisuus kohosi kaksi viikkoa kestäneen hoidon jälkeen myös aivoissa johtaen aspartyyliglukosamiinikertymän osittaiseen alenemiseen. Lisäksi tutkimuksessa havaittiin, että AGU-hiirten kudoksissa aivoja lukuun ottamatta oli merkittäviä määriä mannoosia sisältävää aspartyyliglukosamiinia ($Man_2GlcNAc_2$-Asn), jota on aiemmin löydetty myös AGU-potilaiden virtsasta ja maksasta. $Man_2GlcNAc_2$-Asn -pitoisuuden havaittiin kohoavan AGU-hiirten perna- ja maksakudoksessa iän myötä. Glykosyyliasparaginaasi-hoito alensi tehokkaasti myös $Man_2GlcNAc_2$-Asn -kertymää. Tulokset rohkaisevat kokeilemaan pidempikestoisempaa entsyymikorvaushoitoa vastasyntyneillä AGU-hiirillä, joiden veriaivoeste ei ole vielä täysin kehittynyt. Näin ollen entsyymin pääsy aivoihin olisi tehokkaampaa.

Väitöskirjan toisessa osassa vertailtiin glykosyyliasparaginaasin ja rakenteellisesti sen kaltaisen entsyymin, asparaginaasin, entsymaattisia ominaisuuksia. Glykosyyliasparaginaasi-entsyymin on aiemmin todettu pilkkovan aspartyyliglukosamiinin lisäksi β-aspartyylipeptidejä, joten niitä kokeiltiin myös asparaginaasin substraatteina. Bakteereista eristettyjä asparaginaaseja käytetään akuutin lymfaattisen leukemian (ALL) hoidossa, mutta niiden käyttö aiheuttaa vakaviakin sivuvaikutuksia. Tutkimus osoitti, että leukemialääkkeinä käytetyt bakteeriperäiset asparaginaasit kykenevät pilkkomaan viittä testatuista β-aspartyylipeptideistä, joten osa asparaginaasi-terapian aiheuttamista sivuvaikutuksista saattaa johtua β-aspartylpeptidien pilkkoutumisesta.

Glykosyyliasparaginaasilla on asparaginaasi-entsyymin kaltaisia ominaisuuksia, koska se kykenee pilkkomaan asparagiinia aspartaatiksi. Kyseinen ominaisuus kannusti kokeilemaan glykosyyliasparaginaasia mahdollisena leukemialääkkeenä. Soluviljelytutkimus osoitti, että glykosyyliasparaginaasi tuhoaa B-tyypin ALL-leukemiasoluja yhtä tehokkaasti kuin leukemialääkkeinä käytetyt asparaginaasit. T-tyypin ALL-leukemiasolut puolestaan olivat resistentimpiä kaikille kokeilluille entsyymeille, mutta glykosyyliasparaginaasi aiheutti ohjelmoitua solukuolemaa, apoptoosia, myös niissä.

Tämä väitöskirjatutkimus antaa uutta tietoa glykosyyliasparaginaasin entsymaattisista ja terapeuttisista ominaisuuksista. Tulosten perusteella ihmisperäistä glykosyyliasparaginaasia voitaisiin mahdollisesti käyttää osana AGU- ja leukemiapotilaiden hoitoa.

Luokitus: QU 55, QU 135, WB 330, WD 105, WH 250
Yleinen suomalainen asiasanasto: entsyymikorvaushoito; entsyymit; aspartyyliglukosaminuria; rekombinanttiproteiinit; koe-eläimet; hiiret; leukemia; soluviljely; ohjelmoitunut solukuolema

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Eira Kelo

List of the original publications

This dissertation is based on the following original publications:

I Dunder U, Kaartinen V, Valtonen P, Väänänen* E, Kosma V-M, Heisterkamp N, Groffen J and Mononen I. Enzyme replacement therapy in a mouse model of aspartylglycosaminuria. *The FASEB Journal 14: 361-367, 2000.*

II Kelo E, Dunder U and Mononen I. Massive accumulation of $Man_2GlcNAc_2$-Asn in non-neuronal tissues of glycosylasparaginase-deficient mice and its removal by enzyme replacement therapy. *Glycobiology 15(1): 79-85, 2005.*

III Kelo E, Noronkoski T, Stoineva IB, Petkov DD and Mononen I. β-Aspartylpeptides as substrates of L-asparaginases from *Escherichia coli* and *Erwinia chrysanthemi*. *FEBS Letters 528(1-3): 130-132, 2002.*

IV Kelo E, Noronkoski T and Mononen I. Depletion of L-asparagine supply and apoptosis of leukemia cells induced by human glycosylasparaginase. *Leukemia 23: 1167-1171,* 2009.

*Väänänen is the maiden name of Eira Kelo.

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Contents

Abbreviations

AL	acute leukemia
ALP	alkaline phosphatase
AGU	aspartylglycosaminuria
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
ASAT	aspartate aminotransferase
L-Asn	L-asparagine
L-Asp	L-aspartic acid
ASNase	asparaginase
ASNS	asparagine synthetase
BBB	blood-brain-barrier
BMT	bone marrow transplantation
CmCys	S-carbonylmethyl-L-cysteine
CNS	central nervous system
DAPI	4',6-diamidino-2-phenylindole
DONV	5-diazo-4-oxo-L-norvaline
EcAII	*E. coli* asparaginase
ErAII	*Erwinia chrysanthemi* asparaginase
EBV	Epstein Barr Virus
ER	endoplastic reticulum
ERT	enzyme replacement therapy
FMOC	9-fluorenylmethyl chloroformate
FPLC	fast protein liquid chromatography
GA	glycosylasparaginase
GlcNAc-Asn	β-aspartylglucosamine, 2-acetamido-1-N-(β-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine
HPLC	high-performance liquid chromatography
IC_{50}	the concentration of a drug that is required for 50% inhibition of cell growth
K_m	Michaelis constant; the substrate concentration at which reaction velocity is half its maximal
LSD	lysosomal storage disease
Man	mannose
$Man_2GlcNAc_2$-Asn	α-D-Man-(1→6)-β-D Man (1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→N)-Asn
M6P	mannose-6-phosphate
MDH	malate dehydrogenase
MPR	mannose phosphate receptor
MPS	mucopolysaccharidosis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADH	nicotinamide adenine dinucleotide
Ntn hydrolase	N-terminal nucleophile hydrolase

PCD	programmed cell death
PCT	pharmacological chaperone therapy
PITC	phenylisothiocyanate
SRT	substrate reduction therapy
TdT	terminal deoxynucleotidyl transferase
TMR	tetramethylrhodamine
TUNEL	TdT-mediated dUTP nick-end labelling
V_{max}	maximal reaction velocity

1 Introduction

Glycoproteins have diverse roles in many biological functions, such as host-pathogen interactions, protein targeting and cell-to-cell contacts. In addition, a variety of glycoproteins serve as enzymes, hormones, immunological proteins and structural molecules (Spiro 2002). Glycoproteins are polypeptides with covalently linked carbohydrate moieties. According to their structure, glycoproteins are divided into two main groups. The O-linked glycoproteins have the oligosaccharide chain covalently linked to hydroxyl group (-OH) of serine or threonine of protein whereas in N-linked glycoproteins the oligosaccharides are linked to the amino group ($-NH_2$) in the asparagine of peptide chain.

All N-linked glycoproteins contain a common pentasaccharide core structure consisting of three mannose residues and two GlcNAc units attached to asparagine of the polypeptide (Fig. 1). N-linked glycoproteins are further divided into three major families. High-mannose type glycoproteins contain various amounts of mannose residues outside the core unit whereas in hybrid type N-glycoproteins, the outer oligosaccharide chain contains a variety of sugar and amino sugar structures. The complex type N-linked glycoproteins have also sialic acids in the oligosaccharide chain (Kornfeld 1985). The folding, localization, immunogenicity and bioactivity of a glycoprotein are dependent on its oligosaccharide structure (Moremen 2002).

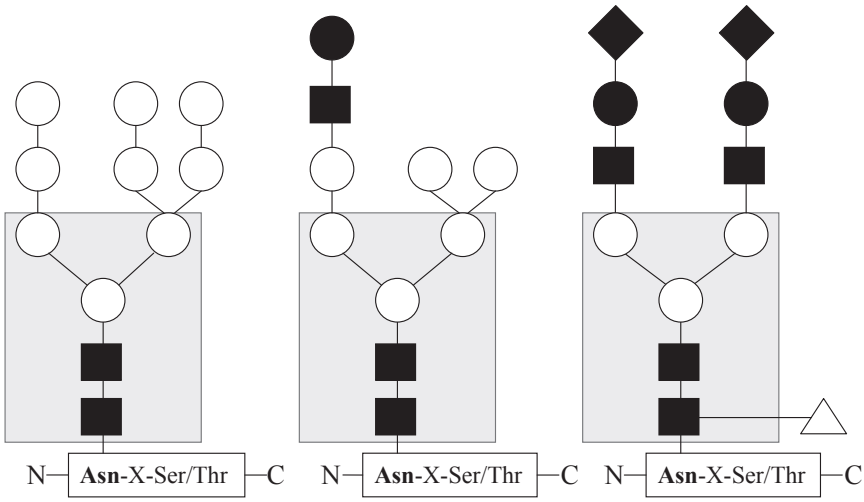


Figure 1. The structure of high-mannose, hybrid and complex type asparagine-linked glycoproteins. The pentasaccharide core is marked with a grey area. The schematic components of glycoproteins are: N-acetyl-β-D-glucosamine (■); α-L-fucose (△); α- or β-D-mannose (○); β-D-galactose (●) and N-acetyl-α-neuraminic acid (◆).

Glycoproteins are degraded to monosaccharides and amino acids in the lysosomes in a bidirectional pathway by over 50 different lysosomal hydrolases. Another set of enzymes, exoglycosidases, cleave the monosaccharides sequentially from the non-reducing end of the glycoproteins (enzymes 1-6 in Fig. 2). The protein region around the N-glycosidic linkage is initially hydrolyzed by proteases, called cathepsins (I). Then, the fucose residue, if present, is cleaved by α-L-fucosidase (II). The third enzyme in the stepwise hydrolysis of the protein-to-

carbohydrate linkage region is glycosylasparaginase (III), which catalyzes the hydrolysis of β-amide of the Asn-GlcNAc linkage. The release of the reducing end GlcNAc from the di-N-acetylchitobiose core structure is hydrolyzed either by chitobiase (IV) or N-acetyl-β-D-glucosaminidase (β-hexosaminidase, enzyme 6) (Aronson 1989) (Fig. 2).

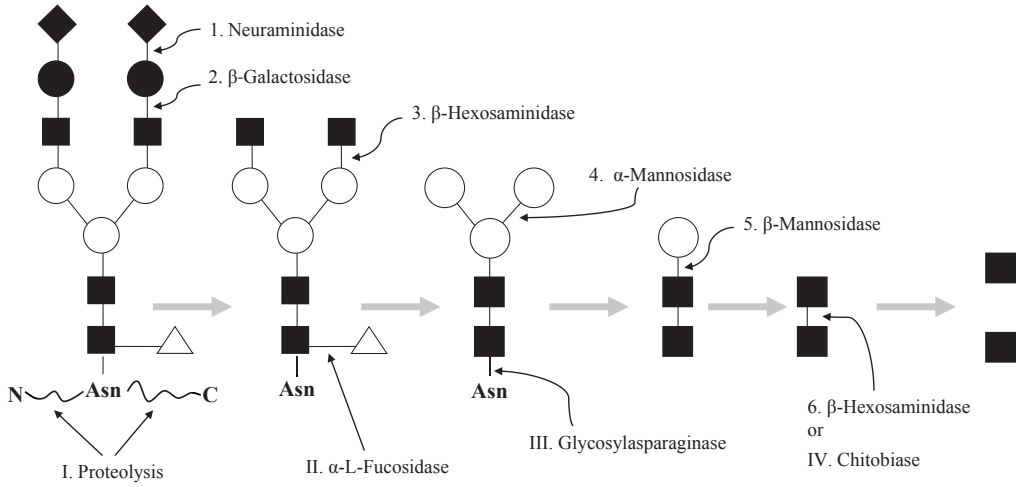


Figure 2. The degradation of Asn-linked glycoproteins in lysosomes. The schematic components of glycoproteins are: N-acetyl-β-D-glucosamine (■); α-L-fucose (△); α- or β-D-mannose (○); β-D-galactose (●) and N-acetyl-α-neuraminic acid (◆).

Sometimes one of the specific lysosomal enzymes is totally or partially absent thus causing an error in glycoprotein degradation. As a result, incompletely degraded glycoproteins can accumulate inside the lysosomes causing many secondary effects such as oxidative stress, ER stress, defects in autophagy, altered calcium homeostasis and a significant energy imbalance (Hawkins-Salsbury 2011) ultimately leading to slowly or rapidly progressive symptoms and death of the patients even in early childhood. The disorders of glycoprotein degradation, the corresponding defective enzyme and accumulated products are listed in Table 1. All the diseases listed in Table 1 are inherited in an autosomal recessive manner.

Aspartylglycosaminuria (AGU, McKusick 208400), the most common disorder of glycoprotein degradation, is attributable to the deficient activity of glycosylasparaginase (GA). The incomplete degradation of glycoasparagines leads to a massive accumulation of aspartylglucosamine (GlcNAc-Asn) and other glycoproteins into tissues and body fluids of the patients. The clinical features of AGU proceed slowly in childhood, but during the later course of the disease, rapid psychomotor retardation occurs usually leading to death before the age of 40 years. AGU is more common in Finland than in other parts of the world and thus it belongs to the Finnish disease heritage (Aula 2001, Arvio 2002).

α-Mannosidosis is divided into the infantile phenotype (type I) and the juvenile–adult phenotype (type II) according to its clinical manifestations and progression. Deficiency of functional α-mannosidase (LAMAN; EC 3.2.1.24) leads to an accumulation of the trisaccharide Man-α-(1-3)-Man-β-(1-4)-GlcNAc and other oligomannosides to tissues and urine of the patients (Michalski 1999, Caeyenberghs 2006).

β-mannosidosis, which is caused by deficiency of the enzyme β-mannosidase (MANBA, EC 3.2.1.25), is very rare; only approximately 20 human cases have been described this far throughout the world and a wide range of symptoms of varying degree of severity is observed. The lack of the β-mannosidase enzyme causes accumulation of different oligosaccharides

consisting of the disaccharide Man(β1-4)GlcNac and other more complex oligosaccharides (Michalski 1999).

Sialidosis (mucolipidosis type I) is caused by the deficiency of lysosomal sialidase (alpha-N-acetyl neuraminidase-1, NEU1, EC 3.2.1.18) and it is classified into two main clinical variants: type I, the milder form of the disease, and type II, which can in turn be subdivided into three forms: congenital, infantile and juvenile. Excessive amounts of oligosaccharides containing N-acetylneuraminic acid at the nonreducing terminus occur in the urine of patients (Beaudet 1989).

Fucosidosis results from a deficiency of α- L-fucosidase (AFU; EC 3.2.1.51) and leads to the accumulation of fucose-containing glycolipids and glycoproteins in many tissues and urine of the patients. The major glycopeptide found in urine is Fuc(α1-6)GlcNacβ1-Asn, which can be explained by the steric inhibition of the glycosylasparaginase by the fucose residue. A fatal infantile form with early mental and motor regression is referred as type I and the milder phenotype with adult survival is designated type II (Kau 2011).

Galactosialidosis is caused by a combined deficiency of lysosomal β-galactosidase and neuraminidase, due to a primary defect in protective protein/cathepsin A (PPCA). PPCA is an intralysosomal protein that protects both β-galactosidase and α-neuraminidase from premature proteolytic processing. Three subtypes of galactosialidosis are recognized: the early infantile type, the late infantile type, and the juvenile/adult type (Coutinho 2012a).

α-N-acetylgalactosaminidase (α-NAGAL, EC 3.2.1.49) cleaves terminal α-N-acetylgalactosamine residues from glycopeptides and glycolipids, primarily O-linked sugars attached to serine and threonine residues. A deficiency of α-NAGAL activity results in the accumulation of glycolipids and glycopeptides in Schindler and Kanzaki patients, who suffer from neurological and other pathologies (Clark 2009).

Table 1. Disorders of glycoprotein degradation

Disorder	Deficient enzyme	Accumulating product	Reference
Aspartylglycosaminuria	Glycosylasparaginase (aspartylglucosaminidase)	Aspartylglucosamine and other N-linked oligosaccharides	Aula 2001
α-Mannosidosis	α-Mannosidase	Man-α-(1-3)-Man-β(1-4)-GlcNAc and other unbranched oligosaccharides	Thomas 2001
β-Mannosidosis	β-Mannosidase	Man-β-(1-4)-N-acetylglucosamine	Thomas 2001
Sialidosis (mucolipidosis I)	Sialidase (neuraminidase)	Sialyl glycoconjugates	Thomas 2001
Fucosidosis	α-L-Fucosidase	Fuc-α-(1-2)-Gal-β-(1-4)-GalNAc-α-Gal-ceramide	Thomas 2001
Galactosialidosis	β-Galactosidase, neuraminidase and protective protein/cathepsin A	Sialyl glycoconjugates	d'Azzo, 2001
Schindler/Kanzaki disease	α-N-Acetylgalactosaminidase	Glycopeptides and oligosaccharides containing α-N-acetylgalactosaminyl moieties	Clark 2009

The above mentioned diseases belong to the glycoproteinoses, a subgroup of lysosomal storage diseases, which also includes disorders of lysosomal enzyme phosphorylation and

localization. I-cell disease (mucolipidosis II, ML-II) and Pseudo-Hurler polydystrophy (mucolipidosis III, ML-III) patients have a defective form of the enzyme N-acetylglucosamine-1-phosphotransferase and thus the lysosomal enzymes miss their mannose-6-phosphate recognition marker and are not targeted to lysosomes. As a result, the lysosomal enzymes are secreted into the extracellular medium instead of lysosomes and the affected cells show dense inclusions with storage material. Those enzymes are also present in the serum and body fluids of affected patients. Pseudo-Hurler polydystrophy is a milder form of I-cell disease characterized by a later onset of clinical symptoms and less accumulation of carbohydrates, lipids and proteins in the inclusion bodies (Coutinho 2012b).

A total of approximately 50 lysosomal storage disorders (LSDs) have been identified this far and they are divided either according to the type of the accumulating compound or the affected lysosomal function. Individual LSDs are rare, but as a group, they are one of the most common genetic disorders in children with an incidence of approximately one out of every 7,000-8,000 live births (Hawkins-Salsbury 2011). Signs and symptoms of these inherited LSDs vary widely in severity, even among affected siblings in the same family, and the age of onset ranges from infancy to adolescence. Two thirds of the LSDs affect the central nervous system (CNS) and mental retardation is common in these patients. The severity of the LSD's is closely related to the residual enzyme activity – the lower the residual activity, the earlier the age of onset (Vellodi 2005).

At present, there is no cure for LSDs but progress has been made in developing effective therapies to reduce the clinical symptoms and to improve the quality of life of the patients. One general goal of the therapy is to lower the excessive amount of storage material through improved substrate degradation, which can be achieved either by hematopoietic cell transplantation (HCT) or enzyme replacement therapy (ERT). HCT has proven to be effective in MPS I (Hurler disease), α-mannosidosis and Krabbe disease patients, if the procedure is performed during early childhood (Beck 2010). However, the major disadvantages of HCT are infection, rejection and graft versus host disease (GVHD) which remain the major causes of morbidity and mortality following the transplantation (Turbeville 2011). In contrast, ERT is generally well tolerated (Beck 2010) and even if the patients develop antibodies against therapeutic enzyme in the beginning of therapy they have shown immune tolerance to the recombinant enzyme after several weeks of treatment.

Enzyme replacement therapy replaces the missing or malfunctioning enzyme by frequent injections of a recombinant enzyme. The intention is to reduce the accumulating compound from tissues and body fluids. The Gaucher's disease was the first lysosomal storage disease successfully treated with ERT and currently there is ERT commercially available for Fabry's disease, mucopolysaccharidoses (MPS) I, II and VI, as well as for Pompe's disease throughout the world (Rohrbach 2007). All of those abovementioned diseases are non-neuronopathic variants of lysosomal storage diseases and naturally the accumulated substrate is more easily obtainable for the enzyme than in neuronopathic LSD's, such as disorders of glycoprotein degradation, which face the problem of penetration through the blood-brain-barrier (BBB) before the enzyme can exert its therapeutic effect.

Before ERT is available for the patients, the preclinical studies need to be done either in cell culture (*in vitro*) or with animal models. For some glycoproteinoses, such as fucosidosis and α-mannosidosis, there exist natural animal models but many of the models have been created by so-called knockout techniques. Among glycoproteinoses, there is a knockout mouse model available for aspartylglucosaminuria, α- and β-mannosidosis, sialidosis and galactosialidosis. Animal models which exactly mimic the LSDs have helped in studying the potential therapies for those disorders. Animal models for glycoproteinoses – both knockout and natural ones - are listed in Table 2.

AGU fibroblasts and lymphoblasts have helped to elucidate the efficacy of enzyme replacement therapy (ERT) *in vitro*. For example, recombinant human glycosylasparaginase has

proven to be effective in correcting the GlcNAc-Asn accumulation in lymphocytes from an AGU patient (Mononen 1995). The mouse models of AGU (Kaartinen 1996, Jalanko 1998) have been useful tools when investigating gene therapy and bone marrow transplantation (BMT) as potential therapeutic approaches for AGU *in vivo*. In the mouse model of AGU, a pattern of accumulating urinary glycoproteins has been described (Jalanko 1998), yet in tissues their exact amounts has not been studied. ERT with AGU has previously been completed only *in vitro*, in cultured AGU lymphoblasts and fibroblasts (Mononen 1995).

Table 2. Animal models for glycoproteinoses

Glycoproteinosis	**Animal model**	**Reference**
Aspartylglucosaminuria	mouse (knockout)	Kaartinen 1996 Jalanko 1998
α-Mannosidosis	mouse (knockout) cat (natural) guinea-pig (natural)	Blanz 2008 Vite 2005 Crawley 2006
β-Mannosidosis	mouse (knockout) bovine (natural)	Zhu 2006 Jones 1993
Sialidosis	mouse (knockout)	de Geest 2002 Zanoteli 2010
Fucosidosis	dog (natural)	Kondagari 2011
Galactosialidosis	mouse (knockout)	Bonten 2004

In the first part of this study, a previously devised mouse model of AGU (Kaartinen 1996) was used to evaluate whether ERT with human recombinant glycosylasparaginase (GA) would have any effect on the storage of aspartylglucosamine (GlcNAc-Asn) in tissues and body fluids of these mice.

Then, the structure of a glycoasparagine, $Man_2GlcNAc_2$-Asn, which is also present in AGU patients' urine, was characterized and its concentrations in the tissues and urine of untreated AGU mice, and AGU mice treated with human recombinant glycosylasparaginase were analyzed quantitatively. Moreover, the effect of ERT on the amount of $Man_2GlcNAc_2$-Asn in tissues of the treated animals was studied and compared to that of GlcNAc-Asn.

In addition to its principal substrate GlcNAc-Asn, GA is able to hydrolyze high mannose and complex glycoproteins (Kaartinen 1992). Furthermore, it has L-asparaginase activity (Noronkoski 1997) and a tetrameric subunit structure rather similar to L-asparaginases (Oinonen 1995, Swain 1993). This similarity between GA and L-asparaginase, and the observation that GA is able to hydrolyze β-aspartyl peptides (Noronkoski 1998), led to the decision to test those peptides also as substrates of bacterial *E. coli* and *Erwinia* L-asparaginase.

Bacterial L-asparaginases, mainly purified from *E. coli* (EcAII) and *Erwinia chrysanthemi* (ErAII), are used in the treatment of acute lymphoblastic leukemia (ALL). The antileukemic effect of L-asparaginase is based on its ability to deplete the extracellular reservoirs of L-asparagine, which is an essential amino acid to some leukemic cell types. Although bacterial L-asparaginases have proven effective in ALL therapy, there also exist many disadvantages associated with their use. Since the bacterial enzyme is a foreign protein in the human body, a

variety of side-effects arise in the patients (Müller 1998). Part of side effects is related to the glutaminase activity of L-asparaginase, because the absence of glutamine leads to defective nitrogen transportation and insufficient function of liver (Ollenschläger 1988).

In the last part of this study, encouraged by the L-asparaginase activity of GA, and the fact that it does not have any glutaminase activity, the antileukaemic features of human recombinant GA were studied *in vitro* in two different leukemic cell lines that are dependent on an external supply of L-asparagine. Furthermore, the ability of GA to hydrolyze both the intra- and extracellular Asn reservoirs *in vitro* was studied using EBV transformed glycosylasparaginase-deficient lymphoblasts.

2 Review of the literature

2.1 GLYCOSYLASPARAGINASE

2.1.1 Discovery

Lysosomal glycosylasparaginase (aspartylglucosaminidase, EC 3.5.1.26, N4-[β-N-acetyl-D-glucosaminyl]-L-asparaginase, GA) was found in sweet-almond emulsin in 1964 by Lee et al., and it was extracted from guinea pig serum a couple of years later by Makino et al. The lysosomal location of GA was first revealed by Mahadevan and Tappel in 1967, when they purified the enzyme from rat liver and kidney (Mononen 1997). They also hypothesized that the lysosomal activity of glycosylasparaginase might be missing from two mentally retarded siblings who excreted GlcNAc-Asn in their urine. In the very next year, the deficiency of GA was finally confirmed to be responsible for AGU by Pollitt et al., who observed that GA activity was absent from the tissues of those two mentally retarded siblings (Pollitt 1968). After its initial purification from rat liver and kidney in 1967, GA has been purified also from human, bovine, mouse, pig, chicken (Tollersrud 1992), Sf9 insect cells (Liu 1996) and bacteria (Tarentino 1995).

2.1.2 Synthesis and structure

After its synthesis, the inactive single 346-amino-acid GA precursor polypeptide loses its N-terminal signal sequence of 23 amino acids during the entry into the endoplasmic reticulum (ER), and is autocatalytically processed and cleaved into a 27 kDa pro-α-subunit, originating from the N-terminal part of the precursor polypeptide, and a carboxy-terminal 17 kDa β-subunit (Fig. 3) (Saarela 1998). The cleavage of GA precursor polypeptide into pro-α- and β-subunits exposes the Thr206 (also called Thr183) residue, which is essential for the proteolytic activation and enzymatic activity of GA (Saarela 2004a) and has found to be conserved from bacteria to mammals. The cleaved, already active enzyme is first delivered to the Golgi complex, where mannose-6-phosphate (M6P) is added to each oligosaccharide chain of the molecule for lysosomal targeting. Inside lysosomes, GA is further trimmed proteolytically in order that it may take on its final heterodimeric or heterotetrameric structure of 24 kDa α- and 14 kDa β-subunits.

GA was considered to have a monomeric structure until 1989, when Tollersrud and Aronson reported the heterodimeric subunit composition of rat liver glycosylasparaginase. Some years later, the cDNA of GA was cloned revealing that one gene encodes two subunits (Mononen 1997). The human leukocyte enzyme (88 kDA) was found to have a heterotetrameric structure by Kaartinen et al. and the three-dimensional structure of GA was described in 1995 by Oinonen et al. (Oinonen 1995). According to several studies, GA can exist as both a heterodimer and a heterotetramer depending on species and the environmental conditions (Mononen 1997). For example, an insect GA from Sf9 cells occurs both as a $\alpha_2\beta_2$ heterotetramer and a $\alpha\beta$ heterodimer.

The human lysosomal GA has a heterotetrameric structure, also called αββα-sandwich, with dimensions of 50x50x70 Å (Oinonen 1995) (Fig. 4a). The core structure contains a four-stranded and an eight-stranded β-sheet packed against each other, and a layer of α-helixes on the outer side of both of those β-sheets (Fig. 4b). The heterotetrameric GA has a total of four intramolecular disulfide bridges, two of them in the α-subunit (Cys64-Cys69, Cys163-Cys179) and another two in β-subunit (Cys285-Cys306, Cys317-Cys345). The α- and β-subunits are linked by noncovalent forces (Mononen 1997).

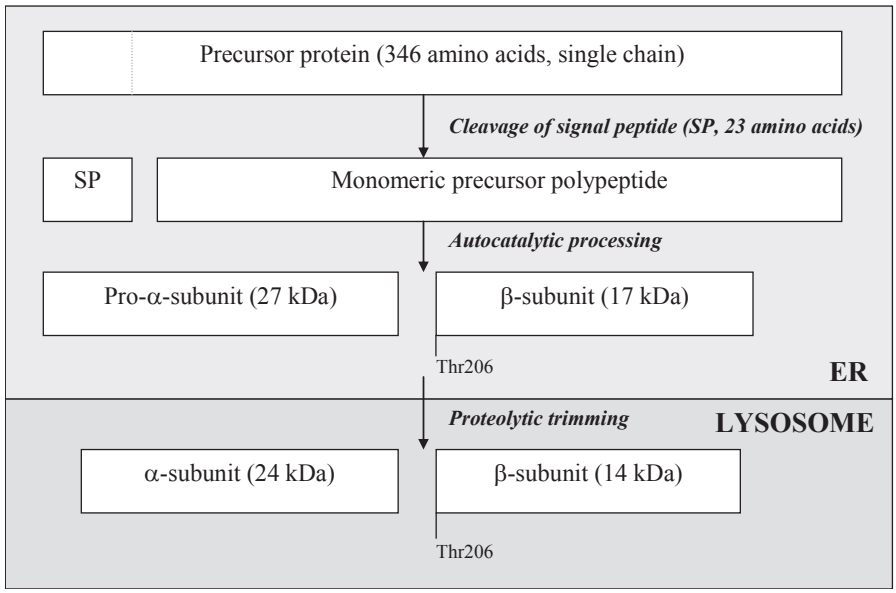


Figure 3. Posttranslational trimming of glycosylasparaginase. ER, endoplastic reticulum; SP, signal peptide

Glycosylasparaginase has two highly conserved glycosylation sites, Asn38 in α-subunit and Asn308 in β-chain. According to monosaccharide analysis of purified human leukocyte glycosylasparaginase it contains both high-mannose and complex type N-glycosidic carbohydrate chains accounting for approximately 15% of the total enzyme weight. GA of human, mouse, pig and cow has been found to be sensitive to endoglycosidase H and alkaline phosphatase, which prevent the transfer of the enzyme into lysosomes by removing either the high-mannose carbohydrate chains or the phosphate residues, respectively (Mononen 1997). A mutation in the β-chain glycosylation site is more important for correct folding and transportation into lysosomes than that of α-peptide (Park 1996). The location or number of the glycosylation sites of GA in different species shows variation; e.g. in the murine β-chain, the carbohydrate is located at Asn310 instead of Asn308, and bovine and rat GA has an additional N-glycosylation site at Asn149 of the α-peptide. Sf9 insect GA has its only glycosylation site in its α-subunit whereas flavobacterium GA does not have any glycosylation sites at all.

Each GA heterotetramer contains two funnel-shaped active sites, which bind the asparagine portion of the substrates at the narrow bottom and the oligosaccharide part near to the wider mouth of the pocket (Oinonen 1995). The binding of aspartylglucosamine to the active site of GA is dependent on the presence of a free α-carboxyl group of aspartylglucosamine, whereas the α-amino group may be substituted with a group of comparable size (Risley 2001). In the active site of human GA, five residues, Arg211, Asp 214, Gly 235, Thr 183 (=Thr206) and Thr 234, of β-chain make direct hydrogen bonds with aspartate (Oinonen 1995) (Fig. 5). The study of *Flavobacterium meningosepticum* glycosylasparaginase-substrate –complex revealed that the binding of the substrate induces a conformational change near to the active site of GA. The catalysis is initiated by polarization of the N-terminal Thr152 side-chain by the free α-amino group on the same residue, mediated by the side-chain hydroxyl group of Thr170. The cleavage of the amide bond is then completed by a nucleophilic attack at the carbonyl carbon of the

amide linkage in the substrate, leading to the formation of an acyl-enzyme intermediate through a negatively charged tetrahedral transition-state (Wang 2007).

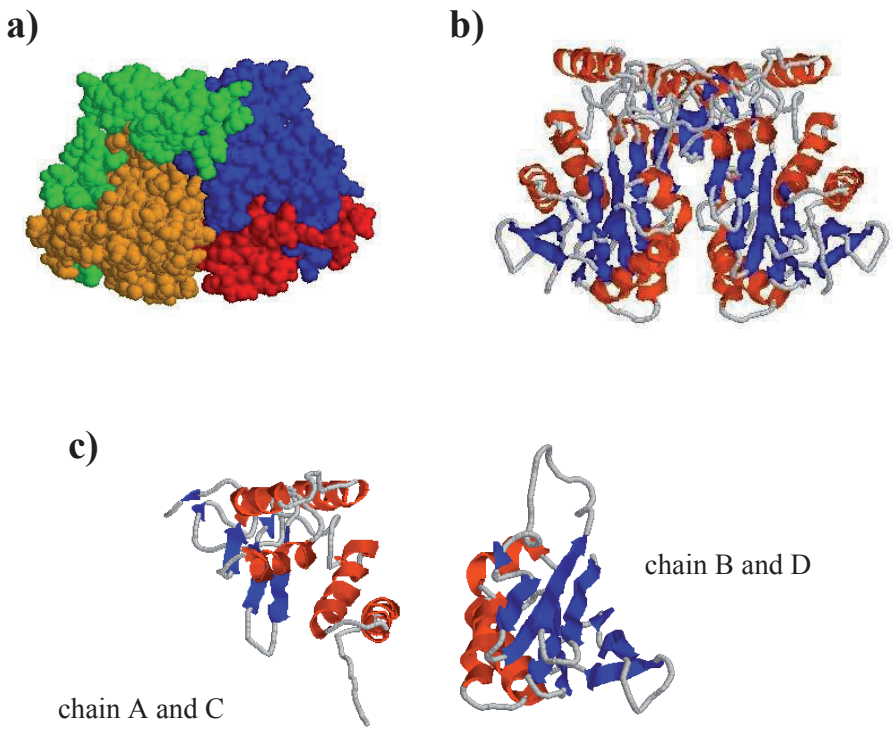


Figure 4. Three-dimensional structure of human glycosylasparaginase (PDBsum code: 1apy). Colours and orientations are modified with RasMol program (Sayle, 1995). a) a spacefill representation of GA. Colour codes showing the four chains are green (A), orange (B), blue (C) and red (D). A cartoon image of the GA heterotetramer (b) and its two different chains (c) showing the secondary structure. Chain A is identical to chain C, and B is identical to D. α-Helices are represented in red, β-strands in blue and loops in grey.

A diazomethylketone analogue of asparagine, 5-diazo-4-oxo-L-norvaline (DONV), is a noncompetitive and irreversible inhibitor of GA due to the formation of an α-ketone ether bond with the N-terminal threonine of the β-subunit (Kaartinen 1991).

2.1.3 Stability, pH optimum, isoelectric point

GA is extremely heat resistant up to 60 °C and it has a wide pH optimum. Table 3 summarizes the pH-optimum, heat stability and isoelectric point of GA of several species.

2.1.4 GA and the other N-terminal nucleophile hydrolases

The family of the N-terminal nucleophile hydrolases (Ntn hydrolases) was first described in 1995 by Brannigan et al. when they reported on the structural similarity of three amidohydrolases i.e. glutamine PRPP amidohydrolase (GAT), penicillin acylase and the

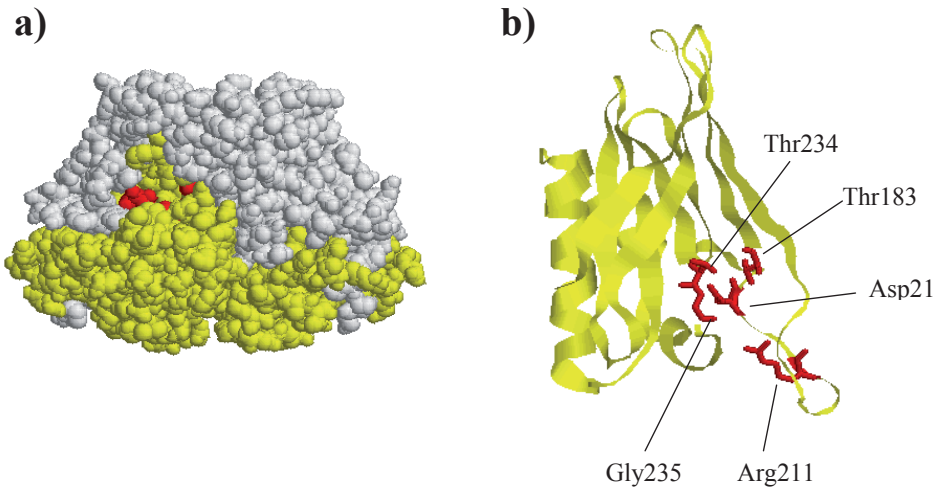


Figure 5. Space fill presentation of GA heterotetramer (a), showing the active site in red (another active site is located on the other side of the enzyme). Chains A and C are presented in white and B and D chains are presented in yellow. Ribbon representation of chain B of GA (b) shows the active site residues of human GA as red sticks

proteasome (Brannigan 1995). The structural superfamily members share the unique αββα-core structure and they all use serine, threonine or cysteine as a nucleophile in the amino-terminal residue of β-sheet which is exposed during the autocatalytic activation. Although there is a strong structural similarity in the Ntn-hydrolases, their amino acid sequences exhibit only a minor homology. Glycosylasparaginase was recognized as the first mammalian member of the family of the Ntn hydrolases by Oinonen et al. (Oinonen 2000). The most recently found mammalian Ntn-family members are human acid ceramidase (Shtraizent 2008) and human asparaginase-like protein 1 (hASRGL1) (Cantor 2009). The currently known members of N-terminal hydrolase superfamily and the corresponding substrates and nucleophiles are summarized in Table 4.

Table 3. pH-optimum, heat stability and isoelectric point of GA in several species

Species	pH-optimum	Heat stability (°C)	Isoelectric point	Reference
Human	liver 5.5 – 6, leukocytes 7 – 9[a]	70	5.0	Tollersrud 1992 [a]Kaartinen 1991 [b]Kaartinen 1992 [c]Noronkoski 1998
Chicken	5.5 - 6	70	6.8	Tollersrud 1992
Pig	5.5 - 6	70	5.1	Tollersrud 1992
Mouse	7 - 8	70	5.3	Tollersrud 1992
Rat	7 - 9	75	5.3-6.3[a], 6.4- 6.6[b]	[a]Tollersrud 1992 [b]Tollersrud 1989
Sf9	4 - 10,5	65-70		Liu 1996
Cow	5.5 - 6	77	6.2	Tollersrud 1992

Table 4. Members of Ntn hydrolase superfamily

Enzyme	Substrate	Nucleophile	Reference
Glycosylasparaginase	GlcNAc-Asn	Thr	Oinonen 2000
Asparaginase (plant)	Asn	Thr	Aronson 1996
Asn synthetase	Gln	Cys	Aronson 1996
Glutamate synthase	2-Oxoglutarate, L-Glutamine	Cys	Kim 1996
Proteasome subunit	Proteins	Thr	Aronson 1996
Cephalosporin acylase	Cephalosporins	Ser/Thr	Kim 2006
Glutamine Phosphoribosyl-pyrophosphate (PRPP) Amidotransferase	Gln	Cys	Kim 1996
γ-glutamyl-transpeptidase	Glutathione	Thr	Okada 2007
GlcN-6-P synthase	Glutamine	Cys	Mouilleron 2011
Acid ceramidase HUMAN	Ceramide	Cys	Shtraizent 2008
Penicillin acylase	Penicillin G	Ser	Aronson 1996
AcylCoA: isopenicillin N acyltransferase	Isopenicillin N	Cys	Bokhove 2010a
Peptidase family U34	Peptides	Cys	Pei 2003
PvdQ	N-Acyl homoserine lactone	Ser	Bokhove 2010b
Lysosomal 66.3 kDa protein	Not known	Cys	Lakomek 2009
hASRGL1 HUMAN	β-Aspartyl peptides	Thr	Cantor 2009

2.1.5 Substrate specificity

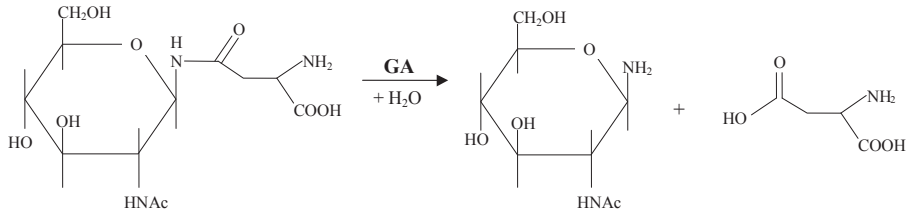
2.1.5.1 Aspartylglucosamine in N-linked glycoproteins

The native substrate of GA is aspartylglucosamine (GlcNac-Asn), which is present in the carbohydrate-to-protein linkage of N-linked glycoproteins. GA catalyzes the hydrolysis of GlcNAc-Asn to 1- amino-N-acetylglucosamine and L-aspartic acid, which are further cleaved nonenzymatically to N-acetylglucosamine and ammonia (Fig. 6).

2.1.5.2 Other substrates

It has been demonstrated that GA is also able to hydrolyze β-aspartylpeptides (isoaspartyl peptides), such as β-aspartylserineamide and β-aspartame (Noronkoski 1998). β-Aspartylpeptides are present in human urine (Kakimoto 1961, Buchanan 1962) and some of them have been found in rat brain tissue (Sandberg 1994). Some of the β-aspartylpeptides are believed to represent the end products of protein degradation (Buchanan 1962) and they are also considered to be of dietary origin (Pisano 1966). In the synthesis and degradation of the β-aspartylpeptides, GA has an important role (Noronkoski 1998).

In addition to GlcNAc-Asn and β-aspartylpeptides, GA is able to hydrolyze non-fucosylated high mannose and complex type glycoasparagines (Kaartinen 1992), aspartyl-N-acetyllactosamine and its sialylated derivatives, as well as L-asparagine (Noronkoski 1997). Table 5 summarizes the substrates of GA and the corresponding reaction rates.



GlcNAc-Asn **GlcNAc-NH_2** **L-aspartic acid**

Figure 6. The hydrolysis of GlcNAc-Asn to GlcNAc-NH_2 and L-aspartic acid by glycosylasparaginase (GA). The reaction products are further hydrolyzed nonenzymatically to GlcNAc and ammonia.

Table 5. The substrate specificity of glycosylasparaginase. ND, not detected.

Substrate	Relative rate	Reference
Asn-Glc	140	Tanaka et al. 1973
Galactosylasparagine (Asn-Gal)	101	Tanaka et al. 1973
N-Aspartylglucosamine (GlcNAc-Asn)	**100**	Kaartinen et al. 1992 Tanaka et al. 1973
Asn-Man	96	Tanaka et al. 1973
β-N-Acetyllactosamine-L-asparagine (GalGlcNAc-Asn)	88	Kaartinen et al. 1992
$Man_9GlcNAc_2$-Asn	85	Kaartinen et al. 1992
β-N-Acetylneuraminyl-α2-3-N-acetyllactosamine-L-asparagine (NeuAcGalGlcNAc-Asn)	71	Kaartinen et al. 1992
β-Aspartylserineamide (β-Asp-Ser-NH_2)	71	Noronkoski et al. 1998
$NeuAc_2Gal_2GlcNAc_4Man_3$-Asn	65	Kaartinen et al. 1992
β-L-Aspartyl methyl ester	63	Kaartinen et al. 1992
β-N-Acetylneuraminyl-α2-6-N-acetyllactosamine-L-asparagine (NeuAcGalGlcNAc-Asn)	53	Kaartinen et al. 1992
Aspartylgalactosamine (GalNAc-Asn)	49	Tanaka et al. 1973
β-Asp-Gly-Val (β-aspartylglycinevaline)	49	Noronkoski et al. 1998
Asparagine (Asn)	41	Noronkoski et al. 1997
Asparagine (Asn)	45	Tanaka et al. 1973
β-Aspartylalanineamide (β-Asp-Ala-NH_2)	35	Noronkoski et al. 1998
β-Asp-Gly-Gln	27	Noronkoski et al. 1998
β-Aspartame (β-Asp-Phe-Ome)	23	Noronkoski et al. 1998
β-Aspartylglycineamide (β-Asp-Gly-NH_2)	15	Noronkoski et al. 1998
β-Aspartyltetraglycine (β-Asp-$(Gly)_4$-OH)	8	Noronkoski et al. 1998
β-Aspartylhydroxamate	7,5	Tanaka et al. 1973
β-Aspartylhydroxamate	4	Kaartinen et al. 1992
β-Aspartylglycine (β-Asp-Gly-OH)	3	Noronkoski et al. 1998
L-Asparthydrazide	0,8	Tanaka et al. 1973
L-Aspartic acid β-(7-amido-4-methylcoumarin)	ND	Mononen et al. 1993

2.1.6 Recombinant human glycosylasparaginase

Recombinant human glycosylasparaginase has been produced in NIH 3T3 mouse fibroblasts (Mononen 1997), CHO-K1 cells (Riikonen 1994) and human leukocytes (Tikkanen 1996). The large-scale production of human recombinant GA in NIH 3T3 mouse fibroblasts showed that

the intracellular level of GA was more than 120-fold than the corresponding activity in nontransfected cells. Furthermore, the purified recombinant enzyme was as active as human leukocyte GA, although its β-chain is 1 kDa smaller than that of native enzyme. Recombinant GA has found to be sensitive to alkaline phosphatase and endoglycosidase H, which prevent the transport of GA into lysosomes by removal of phosphate residues or carbohydrates, respectively (Mononen 1995).

2.1.7 Determination of GA activity

The activity of GA can be detected from biological samples such as blood cells, plasma, serum and cultured fibroblasts by a colorimetric or fluorometric method or by high-performance liquid chromatography (HPLC). The colorimetric detection of GA by the Morgan-Elson reaction is currently not usually performed due to its poorer sensitivity than the other methods. The fluorometric assay uses L-aspartic acid β-(7-amido-4-methylcoumarin) as the substrate and has 40 to 100-fold higher sensitivity than can be achieved with colorimetric method (Mononen 1993). The HPLC assay for detection of GA activity is based on the generation of aspartate from aspartylglucosamine in the reaction catalyzed by GA (Kaartinen 1990).

2.2 ASPARTYLGLYCOSAMINURIA

2.2.1 Discovery

The disease aspartylglycosaminuria (AGU, McKusick 208400) was first described in 1968 by Jenner and Pollitt when they found abnormal amounts of 2-acetamido-1-(β'-L-aspartamido)-1,2-dideoxy-β-D-glucose (aspartylglucosamine) in urine of English siblings suffering from mania and depression (Pollitt 1968). At the same time in Finland, Professor Jorma Palo screened a large number of mentally retarded persons to study the prevalence of phenylketonuria (PKU) in the Finnish population, and discovered an abnormal peptide in the urine of nine patients. He finally identified the unknown peptide as GlcNAc-Asn after reading the report of Jenner and Pollitt. Thereafter, AGU has found to be the most common disorder of glycoprotein degradation. Patients with AGU have been reported in several countries around the world, but in Finland the disease has a much higher prevalence than elsewhere. In Finland, 1-3 AGU children are diagnosed annually. By the end of 2010, a total of 260 AGU patients had been identified in Finland.

2.2.2 Clinical aspects

Diagnosis of AGU is rarely made before the age of one year because the pregnancy usually proceeds without complications and babies appear normal at birth. The early symptoms, such as delayed speech, recurrent respiratory infections, stubborn behaviour and clumsiness are usually diagnosed before the age of five years. At the age of 13-16 years, the skills of AGU patients are at their best and correspond to those of healthy 5-6 -year-old children. However, the course of the disease varies individually. AGU proceeds slowly until the age of 25-28 years, but during the later course of the disease, coarsening of the habitus, as well as rapid mental and motor retardation occurs, and the patients will need continuous help in everyday living. The average lifespan of AGU patients is approximately 40 years (Aula 2001, Arvio 1997, Arvio 2002).

2.2.3 Mutations causing AGU

AGU is an autosomal recessively inherited lysosomal storage disease meaning that the patients have inherited a mutated glycosylasparaginase gene from both of their parents. The human glycosylasparaginase gene was localized to chromosome 4q34-35 in 1992 and since then, more than 25 different mutations, including splicing and missense mutations, insertions and

deletions, have been described, most of them representing rare family AGU alleles (Saarela 2004b). The double point mutation responsible for 98% of the AGU cases in Finland, AGU_{FIN}, is caused by the substitution of cysteine 163 with serine (G488C transition) and substitution of arginine 161 with glutamine (G482A transition). The substitution of cysteine is solely responsible for the AGU since it causes the loss of one disulphide bridge thus preventing the cleavage of the peptide into the active enzyme. The mutated enzyme remains as an inactive and misfolded single chain polypeptide and is rapidly degraded already in ER (Aronson 1999). Substitution of arginine represents a neutral polymorphism and does not affect enzyme activity (Aronson 1999). The minor Finnish AGU allele is a 2-bp deletion in exon 2 of the GA gene, which causes a frameshift resulting in a premature stop codon. Heterozygous AGU carriers have mutation only in one allele, and they do not suffer from clinical symptoms of AGU (Aula 2001). The AGU_{FIN} major gene in Finland has shown a carrier frequency of 1 case in 50–85 (Arvio 2002).

2.2.4 Biochemistry and diagnosis

In AGU patients, the deficient activity of glycosylasparaginase leads to the accumulation of GlcNAc-Asn and other glycoasparagines in body fluids and tissues. Urinary level of aspartylglucosamine can be even 1000-fold higher in AGU patients when compared to healthy controls and carriers of the disease (Mononen 1997). In addition, increased amounts of both neutral and acidic glycoasparagines are detectable in urine (Pollitt and Pretty 1974). A list of the glycoasparagines detected in urine of AGU patients is presented in Table 6.

Table 6. Glycoasparaginases found to be present in urine of AGU patients

Compound	Reference
GlcNAc-Asn	Mononen 1997
Man_2-$GlcNAc_2$-Asn	Pollitt 1974 Lundblad 1976 Gordon 1998
Gal-GlcNAc-Asn	Lundblad 1976
Neutral glycoasparagines containing fucose, mannose or galactose Fuc-Gal-GlcNac-Asn Man_2-$GlcNAc_2$-Asn Man_2-Gal-GlcNAc-Asn	Pollitt and Pretty 1974
Acidic glycoasparagines containing N-acetylneuraminic acid (NANA) NANA-Gal_2-GlcNAc-Asn $NANA_2$-Gal_2-GlcNAc-Asn	
NANA-Gal-GlcNAc-Asn	Sugahara 1977

A traditional but still a useful method for the diagnosis of AGU is the detection of glycoasparagines from urine with thin layer chromatography (TLC), in which the pattern of carbohydrate containing structures can be visualized with either ninhydrin or orcinol. Glycoasparagines have been detected also by electrophoresis and paper chromatography, but currently HPLC offers a more sensitive, quantitative and faster analysis (Mononen 1997).

GA activity in the tissues and body fluids of AGU patients have found to be totally absent or reduced to less than 10% when compared to healthy individuals. Interestingly, the mean GA activity of the AGU carriers is only 25% of the normal lymphocyte activity, but 72% and 75% of normal plasma and serum activity, respectively. Due to the overlap in GA activity between the normal controls and the carriers of AGU, the determination of GA activity is not reliable for carrier detection (Mononen 1994).

The assay of GA activity was initially based on colorimetric detection of the released N-acetylglucosamine by the Morgan-Elson reaction in the presence of aspartylglucosamine, but it was found to be unspecific and the analysis required a long incubation time. Currently, even low levels of GA activity can be determined from serum, plasma, blood cells or fibroblasts of the patient either by a fluorometric assay using L-aspartic acid β-(7-amido-4-methylcoumarin) as the substrate or by HPLC (Mononen 1997).

The carriers of AGU$_{FIN}$ mutation can be detected from blood by three different polymerase chain reaction (PCR) -based methods; oligonucleotide ligation assay, hybridization with allele-specific oligonucleotides and solid-phase minisequencing (Romppanen 1997). Prenatal diagnosis of AGU is based on the assay of GA activity from cultured amniotic cells, chorionic villus samples and amniotic fluid, or the identification of the disease-causing mutation in both alleles of the GA gene (Mononen 1997). Currently in Finland, the diagnosis of AGU is based on DNA methods as well as the detection of urinary oligosaccharides by TLC and HPLC.

2.2.5 Therapy and animal models of AGU

After the discovery of the molecular and cellular mechanisms contributing to AGU, the strategies for effective therapy have been widely studied. Thus far no curative treatment is available, but according to studies performed either *in vitro*, in AGU mouse model or in human patients, enzyme replacement therapy (ERT), bone marrow transplantation (BMT) and gene therapy may be potential therapeutic approaches. Early diagnosis of AGU before the onset of irreversible pathologies, such as skeletal abnormalities, is essential for effective therapy (Meikle 2003).

Two transgenic mouse models of AGU have been generated, one with a targeted disruption of exon 3 (Kaartinen 1996) and another having a mutation in exon 8 (Jalanko 1998). Both AGU mouse models lack the GA activity and share the typical biochemical and phenotypic characteristics of the human AGU disease including the accumulation of GlcNAc-Asn in tissues and urine as well as the slow course of the disease (Gonzalez-Gomez 1998, Jalanko 1998). Mouse models have proved to be useful in both the investigation of the biochemical consequences of the metabolic defect and the development of therapeutic protocols for AGU.

2.2.5.1 Enzyme replacement therapy

In AGU, the *in vitro* studies investigating the efficacy of enzyme replacement therapy have been performed in AGU fibroblasts and lymphoblasts leading to the correction of intracellular GA activity and the restoration of the GlcNAc-Asn concentration to the normal level (Enomaa 1995, Mononen 1995). Additionally, it has been demonstrated *in vitro* that uptake of extracellular recombinant GA is possible also by neuronal cells, indicating that ERT may be effective in brain tissue (Kyttälä 1998). However, the efficacy of ERT has not been evaluated in human AGU patients.

2.2.5.2 Bone marrow transplantation (BMT)

Bone marrow transplantation of eight-week-old AGU mice has shown that BMT is effective in correcting the pathology of liver and spleen, but not that of brain and kidney (Laine 1999). More recent studies with 3-week-old AGU mice have shown that BMT with wild-type donors having normal enzyme activity results in total correction of lysosomal vacuolization in liver and 30-80% reduction of vacuolated neurons in brain (Laine 2004).

BMT therapy in human AGU patients has also been reported, but the benefits of BMT are difficult to predict due to the slowly progressing nature of AGU. A Canadian boy who received BMT at the age of 1.5 years, showed a reduced urinary excretion of GlcNAc-Asn and increased GA activity in white blood cells for several weeks after therapy, but long-term effects of BMT on the neurological symptoms was not reported (Laitinen 1997). Three children carrying the AGU$_{FIN}$ mutation underwent allogeneic BMT at the age of 1.5, 2.0 and 2.6 years, and two of

them showed improvements in radiological, histopathological and biochemical tests. After the follow-up of 3 years, the GA activity in their blood leukocytes was near to that of heterozygous level and storage lysosomes had disappeared from the rectal area. Neurological effect of BMT was followed by magnetic resonance imaging (MRI), and at the end of the follow-up, the ratio of deep grey matter and white matter signal intensities was near to the normal level (Autti 1997). Furthermore, two AGU children underwent allogeneic BMT at the ages of 2 and 2.6 years, and slowly normalization of the pathological, biochemical and MRI findings were found in both of them during the four and seven years' follow-up, but no significant progress was seen in mental level (Autti 1999).

In 2001, Arvio et al. reported that some of the patients, who had undergone transplantation at the age of 1.6-10.4 years, were even more severely retarded than the non-transplanted patients, and several patients experienced from post-transplant complications. Due to the unfavourable outcome of those BMT patients, they concluded that transplantation should be recommended for AGU patients only in infancy to achieve the best beneficial effect on neuropsychological functioning (Arvio 2001). Malm et al. reported the five-year follow-up of two human AGU siblings, the same patients which were included in the study of Arvio et al. Neither of the children had lost any capabilities since transplantation, the activity of GA in leukocytes was normalized and Tau-protein had reached almost normal levels during the study, indicating an arrest of the disease. It was also suggested that BMT may prevent further progress of AGU disease and may have a positive impact on the quality of life as compared to untreated AGU patients (Malm 2004).

Currently, due to the negligible advantages of BMT in the treatment of AGU, it is not routinely used for AGU patients.

2.2.5.3 Gene therapy

In somatic gene therapy, the patient's own cells can be genetically modified *in vitro* or *in vivo* to express the correcting enzyme (D'Azzo 2003). At the present time, gene therapy of AGU has only been tested *in vitro* and in the mouse model of AGU. After a retroviral *in vitro* infection, a significant, 40-fold, increase in GA activity was achieved in AGU fibroblasts when compared to untreated AGU cells, and the activity remained high for at least two months (Enomaa 1995). A few years later, Peltola et al. used adenovirus vectors encoding the human GA in the treatment of AGU mice. A total correction of lysosomal storage after one month, and an increase in GA activity lasting for at least 4 months, was observed in liver by injection of adenoviral vectors into the tail vein of AGU mice. An injection of viral vectors directly into the left lateral ventricle increased GA activity for 4 months in brain tissue. When recombinant GA was injected intraventricularly into AGU mice, the enzyme diffused across the ependymal cell layer lining the ventricles leading to partial correction of lysosomal storage in the adjacent brain tissue. Similar results were achieved with an intraventricular injection of retroviral vectors indicating that even a small amount of transduced cells in brain is able to distribute GA enzyme to the neighbouring areas (Peltola 1998).

Virta et al. used tissue-specific promoters, such as neuron-specific enolase (NSE), in their adenovirus-mediated gene therapy study. The viruses were injected directly into the striatum, which is an area with connections to several brain areas. As a result, the decrease in lysosomal accumulation was evident in wide areas of the brains of the AGU mice (Virta 2006).

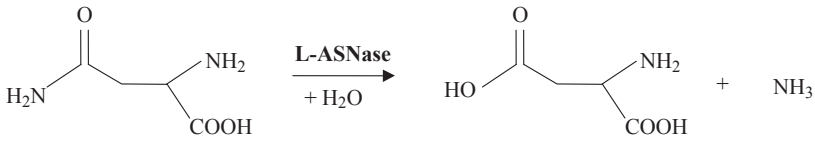
2.3 L-ASPARAGINASES

L-asparaginase (L-asparagine amidohydrolase; EC 3.5.1.1) is an enzyme that primarily catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia (Fig. 7).

2.3.1 Discovery

The hydrolysis of L-asparagine to L-aspartate and ammonia was first described in 1904 by Lang and those results were confirmed by Fürth and Friedmann in 1910. In 1922 Clementi reported that asparaginase is present only in the liver of omnivorous animals, in contrast to herbivores which have asparaginase in practically all tissues. In 1953 Kidd noted that guinea pig serum suppressed the growth of lymphosarcomas in mice, and the antileukemic properties of asparaginase were recognised for the first time by Broome who confirmed that L-asparaginase was the active antitumor component in serum (Broome 1961, Müller 1998, Keating 1993). Further studies proved that leukemic cells require extracellular asparagine for their growth and metabolism, and L-asparaginase isolated from *E. coli* and *Erwinia chrysanthemi* was successively used for clinical trials in childhood acute lymphoblastic leukemia (Müller 1998).

L-asparaginase has been found to be present in chicken liver, yeasts and molds, plants, serum of some rodents, in several bacteria (Howard 1972) and in fungal isolates (Shrivastava 2010), but it is not expressed in humans.



L-Asparagine **L-Aspartic acid** **Ammonia**

Figure 7. Hydrolysis of L-asparagine to L-aspartic acid and ammonia by L-asparaginase

2.3.2 Structure

The classification of all L-asparaginases (Fig. 8) is based on their amino acid sequences (Labrou 2010, Borek 2001). The first class includes the bacterial type L-asparaginases which can further be divided into two subclasses. Bacterial type II L-asparaginases are located in the periplasmic space of bacteria, whereas type I L-asparaginases are cytosolic. Type II L-asparaginases can also hydrolyse L-glutamine and those L-asparaginases which have a greater specificity against L-glutamine are called glutaminase-asparaginases (EC 3.5.1.38). Members of the second subclass exist in plants and are thus named plant-type-L-asparaginases. This group of L-asparaginases displays no homology to bacterial-type asparaginases but it shares approximately 60% of amino acid sequences with the aspartylglucosaminidases, like glycosylasparaginase, which belong to the superfamily of N-terminal nucleophile hydrolases (Hejazi 2002). The structural difference between glycosylasparaginase and plant-type asparaginase is the occurrence of two aromatic residues, Thr-11 and Phe-13, in the vicinity of catalytic Thr in glycosylasparaginase. The third class of L-asparaginases includes *Rhizobium etli* L-asparaginases which exhibit no homology with the other L-asparaginase classes.

Type II bacterial L-asparaginases, such as EcAII and ErAII, are highly conserved homotetrameric enzymes composed of four identical ~330 amino acid subunits, which consist of two α/β domains connected by a linking sequence. The N-terminal domain of each monomer contains an eight-stranded mixed β-sheet, four α-helices and a separate β-hairpin positioned toward the interior of the tetramer. The C-terminal domain is smaller, and has a four-stranded parallel β-sheet and four α-helices (Fig. 9). One unique feature of all L-asparaginases is the left-handed crossover between strands β4 and β5. The amino acid residues present in crossover constitute part of the active site and are evolutionarily conserved. The overall structure of EcAII and ErAII is rigid, with an exception of flexible loop region (residues 14-33 in ErAII), which controls the access of the substrates into the binding pocket (Labrou 2010). The tetrameric enzyme has four independent active sites, which are located between subunits in the intimate dimers (AC and BD). The binding pocket of EcAII contains residues Thr12, Tyr25, Ser58, Gln59,

Thr89, Asp90, Ala114 and Lys162 from one subunit, and Asn248 and Glu283 from the other intimate subunit (Labrou 2010). Water molecules and the hydrogen-bond network formed by the side-chains of the active site residues are important for the activity of the enzyme (Kravchenko 2008).

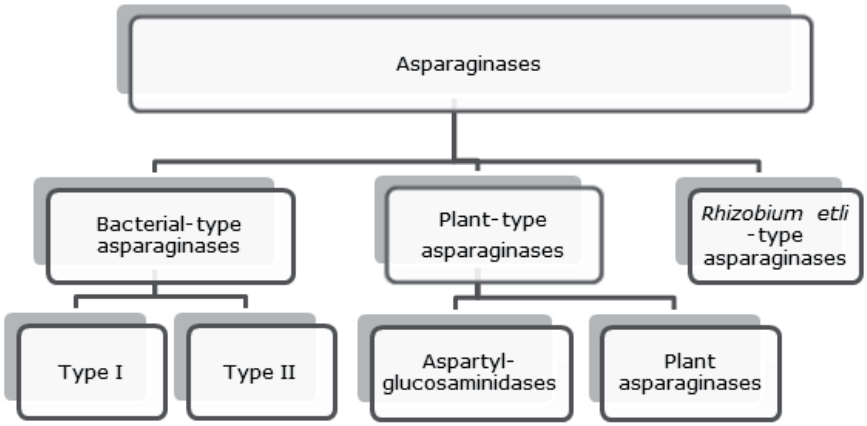


Figure 8. Classification of asparaginases

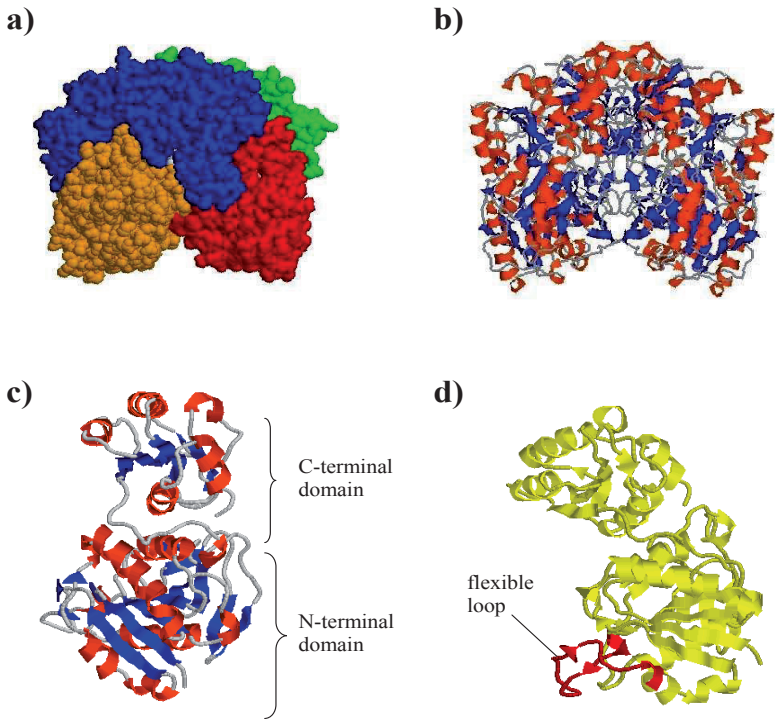


Figure 9. Three-dimensional structure of native L-asparaginase purified from *E. coli* (PDBsum code: 3eca). Colours and orientations are modified with RasMol program (Sayle, 1995). a) The spacefill representation of *E. coli* L-ASNase tetramer. Colour codes for identical subunits are orange (A), green (B), blue (C) and red (D). Cartoon representation of L-asparaginase homotetramer (b) and one subunit (c) showing the secondary structure elements; α-helices are coloured in red, β-sheets in blue and loops in grey. Cartoon representation of the L-ASNase subunit showing the flexible loop (residues 12-33) in red.

Crystallographic structures of several bacterial L-asparaginases, including *E. coli* asparaginase type II (Swain 1993), *Erwinia* asparaginase (Aghaiypour 2001) and *Erwinia carotovora* EwA (Papageorgiou 2008) have been determined, but at present no eukaryotic enzymes have been characterized. The crystal structure of type I bacterial asparaginase was proved to be dimeric by Yao et al. in 2005 (Yao 2005) when this enzyme from *Pyrococcus horikoshii* was characterized. The dimer corresponds to the intimate dimer of EcAII.

2.3.3 Substrate specificity

E. coli asparaginase type II, which is located in the periplasmic space of bacteria, has higher affinity for L-asparagine than the cytosolic asparaginase type I. *E. coli* and *Erwinia* L-asparaginases, EcAII and ErAII, possess glutaminase activity only 2-9% of that of L-asparaginase activity (Table 7) (Howard 1972, Campbell 1967, Campbell 1969).

An isoaspartyl peptidase found in *E. coli* has been shown to have L-asparaginase activity; its amino-acid sequence is homologous to those of plant-type asparaginases and it has a structure similar to that of glycosylasparaginase. The *E. coli* isoaspartyl peptidase undergoes autoproteolysis and has a Thr residue as a nucleophile, thus belonging to a superfamily of N-terminal nucleophile hydrolases, as does the glycosylasparaginase (Prahl 2004). Recombinant plant-type asparaginases from the cyanobacteria *Synechocystis* sp. and *Anabaena* sp. and from the plant *Arabidopsis thaliana* expressed in *E. coli* were able to hydrolyze L-asparagine and β-aspartyl dipeptides, but not GlcNAc-Asn (Hejazi 2002). On the contrary, none of the isoaspartyl dipeptide substrates could be cleaved by type II L-asparaginase from *E. coli*.

In addition to L-asparagine and L-glutamine, L-asparaginases are able to hydrolyze D-asparagine (Campbell 1969), succinamic acid, L-aspartate-β-hydraxamate, L-aspartate-methylester, DL-N^{α}-ethylasparagine and N^4-methoxy-L-asparagine (Herrmann 1974) (Table 7). An asparagine analog 5-diazo-4-oxo-L-norvaline (DONV) is both a substrate and an inhibitor of L-asparaginase (Jackson 1970).

Table 7. Substrate specificity of L-asparaginases purified from *E. coli* and *Erwinia carotovora*

	Relative rate (%)	
Substrate	***E. coli***	***Erwinia***
L-Asparagine	100	100 [a]
D-Asparagine	8 [b]	5 [a]
L-Glutamine	2-3 [b,c]	9 [a]
D-Glutamine		0,02 [a]
Succinamic acid	0 [d]	20 [a]
β-L-Cyanoalanine		5,7 [a]
DL-Isoasparagine		0,1 [a]
L-Aspartohydroxamic acid (L-aspartyl-β-hydroxamate)		19 [a]
N^4-Hydroxyl-L-asparagine	105 [d]	
N^4-Methoxy-L-asparagine	20 [d]	
N^4-Methyl-L-asparagine	0,8 [d]	
N^2-Ethyl-DL-asparagine	2,5 [d]	
Glycyl-L-asparagine	0,5 [d]	

[a] Howard, 1972 [c] Campbell, 1967
[b] Campbell, 1969 [d] Herrmann, 1974

2.3.4 Biochemical analysis

The determination of L-asparaginase activity from biological samples is commonly based on the formation of ammonia from L-asparagine. One unit of asparaginase activity is defined as the

amount of enzyme that liberates 1 μmol of ammonia from asparagine per min at 37 °C. The liberated ammonia can be detected with a spectrophotometer by using Nessler's reagent (Jackson 1970, Boos 1996, Campbell 1967), Berthelot reaction (Schwartz 1970) or with an ammonia gas-sensing electrode (Tagami 1990). Nessler's reagent, a solution of potassium tetraiodomercurate(II) in potassium hydroxide, turns to a yellow color in the presence of ammonia and at higher concentrations, a brown precipitate may be formed. Berthelot's reagent, which is an alkaline solution of phenol and hypochlorite, forms a blue product in the presence of ammonia.

L-asparaginase activity can also be determined by an enzyme coupling method, in which the rate of the decline in the absorbance that occurs as a result of oxidation of NADH is measured spectrophotometrically (Asselin 1993, Tsurusawa 2004). The hydrolysis rate of L-asparagine can also be followed by an amino acid analyzer (Howard 1972) or HPLC (Noronkoski 1997). In addition to L-asparagine, alternative compounds such as AspAMC in a fluorometric L-asparaginase assay (Ylikangas 2000) or 14C-labelled L-asparagine (Broome 1968) and L-aspartic β-hydroxamate (AHA) (Lanvers 2002) can be used as the substrate of L-asparaginase.

2.3.5 Asparaginase in treatment of leukemia

2.3.5.1 Acute leukemias (AL)

Acute lymphoblastic leukemia (ALL) is the most common cancer in children but it can occur at all ages. In ALL patients, the genetically altered hematopoietic stem cells or their committed progenitors undergo dysregulated proliferation and the malignancies arise from lymphoblasts, either B- or T-cells. It has been claimed that most of the childhood ALL cases have a prenatal origin, and an additional exposure, such as an infection, is required for the initial step in the cascade of biochemical events leading to genetic alterations and finally to leukemia (Taub 2004, Cazzaniga 2011).

Due to the advanced molecular and immunological techniques available, ALL can be divided into various subtypes according to the cytomorphology, immunophenotype and genetic information of the leukemic cells (Bacher 2009). During the last decade, microarray-based genomic profiling (Simons 2011), fluorescence in-situ hybridization (FISH), polymerase chain reaction (PCR) and candidate gene sequencing or next-generation sequencing (NGS) (Bacher 2009, Grossmann 2011, Atak 2012) have been used in the characterization of ALL. The characterization of the ALL subtype by using a combination of several methods is essential for the stratification of patients into distinct prognostic risk groups and the planning of optimal treatment (Grossmann 2011).

As one example, TEL-AML1 gene fusion, which is the most common B-lineage ALL phenotype, has a favourable outcome, partly due to its sensitivity to L-asparaginase (Loh 2006) whereas infant ALL with mixed lineage leukemia (MLL) rearrangement still remains a challenge to therapy (Pui 2010). In Finland, approximately 60 new cases of ALL occur in children each year and most of them have the Pre-B-ALL subtype. Finnish laboratories use cytomorphology, surface antigen studies, cytogenetic techniques and PCR to determine the ALL subtypes of the patients.

The outcome of pediatric ALL patients has improved significantly over past decades in the developed countries and currently more than 85% of children with ALL will be leukemia free at 5 years (Pui 2012). On the contrary, the long-term disease-free survival rate among adults is significantly lower, only approximately 40% (Patil 2010). Some of those ALL subtypes which previously had a poor outcome, currently have a much better outlook. For example, the prognosis of ALL patients positive with Philadelphia chromosome (BCR–ABL1) has radically improved since the introduction of tyrosine kinase inhibitors (TKIs) in the therapy of these patients (Lee 2011).

Acute myeloid leukemia (AML) is the most common acute leukemia diagnosed in adults (Patel 2012) and its incidence will likely increase along with the aging population (Mawad 2012). According to current knowledge, AML arises from the cooperation between two classes of genetic alterations that regulate self-renewal and differentiation (Pui 2011). Thus, AML is a heterogenous disease which can be categorized into distinct clinicopathologic subgroups on the basis of their molecular genetic defects (Döhner 2010, Roboz 2011). The 5-year event-free survival for AML in developed countries currently ranges between 49% and 63%.

In the near future, there is a hope for further improvements in the cure rate for acute leukemias if the genetic basis of leukemogenesis can be characterized. Since this will help to understand and predict treatment failure and to provide novel markers for diagnostic testing and targeting of novel personalized therapies (Mullighan 2012, Pui 2012).

2.3.5.2 Asparaginases in clinical use

Since the 1970's, L-asparaginase has been widely used in the treatment of ALL in combination with other drugs. The mechanism of its action is based on the dependence of leukemic lymphoblasts on extracellular L-Asn. Leukemic cells express only low levels of the asparagine synthetase (ASNS) gene and thus they have a very poor capability to produce their own asparagine supply. Thus, the presence of L-asparaginase during the intensive chemotherapy leads to depletion of L-Asn reservoirs and this affects the asparagine-dependent protein synthesis ultimately resulting in death of the leukemic cells (Iwamoto 2007, Chen 2011).

During the last decade, L-asparaginase has been found to be effective also in the treatment of AML (Rubnitz 2009, Buaboonnam 2013). Currently, L-asparaginase is used in children in AML treatment protocol such as ELAM 02 2010. Especially the blast cells of AML subtypes M1, M4 and M5 have been demonstrated to be sensitive to L-asparaginase due to their remarkably low level of ASNS (Okada 2003). Furthermore, L-asparaginase might be a key candidate drug for patients with myeloid/natural killer cell precursor acute leukemia (Morimoto 2010).

There are currently three asparaginase preparations available for clinical use; native enzyme purified from *E. coli* (Elspar®, Medac), polyethylene glycol -conjugated *E. coli* asparaginase (PEG-asparaginase, pegaspargase, Oncaspar®) and native enzyme from *Erwinia chrysanthemi* (Erwinase®) which is antigenically distinct from the *E. coli* enzyme. Asparaginase therapy is generally started with native *E. coli* ASNase, but if this form is not tolerated, it may be changed to PEG-asparaginase. In UK, all children with ALL receive Oncaspar® (Patel 2009). PEG-asparaginase has proved to be effective and requires less frequent injections than the *E. coli* and *Erwinia* asparaginases due to its longer half-life (Graham 2003). In case of development of clinical allergies to native *E. coli* asparaginase or PEG-asparaginase, a switch to *Erwinia* L-ASNase is recommended (Raetz 2010). In Finland, PEG-asparaginase is used unless a switch to *Erwinia* asparaginase is needed due to allergic reactions.

One promising formulation of L-asparaginase is GRASPA®, a suspension containing homologous human red blood cells loaded with L-asparaginase as an active component and SAG-mannitol as a preservative. In the blood circulation, red blood cells act like micro-size bioreactors pumping L-asparagine from plasma to the erythrocytes, in which the asparagine is cleaved by L-asparaginase. The results of the GRASPALL 2005-01 trial, a phase I/II study in children and adults with ALL in first relapse, showed that one single 150 iu/kg GRASPA® dose was as effective as eight injections of 10000 iu/m^2 of native *E. coli* L-asparaginase. In addition, GRASPA® evoked fewer side effects than the native enzyme. Furthermore, the half-life of GRASPA® was as long as 40 days and one dose maintained the asparagine depletion for 18.5 days (Domenech 2011). At present (the beginning of 2013) there are two studies ongoing in Europe with GRASPA®, a phase II/III study in relapsed and allergic ALL patients and a phase II clinical trial as a first line treatment in adults.

2.3.5.3 Side effects caused by L-asparaginase therapy

The majority of ALL patients suffer from side-effects such as pancreatitis, thrombosis, central nervous system complications, liver dysfunction, allergic reactions and even severe anaphylactic shock during L-asparaginase therapy. Especially teenage and adult ALL patients are more likely to suffer from side-effects (Avramis 2006) severe enough to limit the use of high-dose L-asparaginase therapy. Hypersensitivity is the most common toxicity caused by L-asparaginase therapy with a frequency of up to 30% in ALL patients. The reason for hypersensitivity is the development of anti-asparaginase antibodies against the bacterial proteins foreign to the human body, which may shorten the half-life of asparaginase and decrease its activity. In some cases, a phenomenon called silent hypersensitivity arises when circulating antibodies neutralize asparaginase without causing any clinical symptoms of hypersensitivity (Raetz 2010).

The toxicity of *E. coli* and *Erwinia* asparaginases is also related to their L-glutaminase activity, which affects the nitrogen transportation in blood and exerts biochemical disorders especially in liver (Ollenschläger 1988, Krasotkina 2004). If asparaginase depletes both asparagine and glutamine, then asparagine synthetase is not able to produce enough asparagine from aspartate and glutamine. Reinert and colleagues studied tissue-specific nutrient stress in mice, demonstrating that *E. coli* asparaginase abolished circulating levels of asparagine and glutamine, resulting in reduced protein synthesis in liver and spleen but not in pancreas. On the contrary, glutaminase-free asparaginase from *Wolinella succinogenes* did not provoke any changes in protein synthesis in any tissues (Reinert 2006).

Several approaches have been tested to decrease the activity of asparaginase against glutamine. Engineering of substrate specificity by site-directed mutagenesis (Derst 2000) and in silico mutagenesis (Offman 2011) have demonstrated that glutaminase activity can be minimized and the corresponding asparaginase activity even increased. In addition, novel L-asparaginase sources with negligible glutaminase activity have been identified (Kumar 2011, Dhavala 2009). Recently, however, it was shown by Offman that some glutaminase activity is required in order to maintain the optimal activity of *E. coli* asparaginase. Offman demonstrated that glutaminase activity can be reduced at least to half of the wild type activity without affecting the overall asparaginase activity, but a glutaminase-free asparaginase exhibited significantly reduced asparaginase activity. It remains to be determined whether other bacterial asparaginases also need some glutaminase activity in order to maintain their efficacy as leukemia drugs.

2.3.5.4 Resistance to asparaginase

The biologic half-lives of clinically used native *Erwinia* L-asparaginase and *E. coli* L-asparaginase are rather short 0.65±0.13 days and 1.44±0.35 days, respectively, when compared to PEG-conjugated asparaginase which has a half-life of 5.73±3.24 days (Raetz 2010). PEGylation of asparaginase increases its molecular size and structural stability thus improving the plasma half-life and protecting the protein against proteolytic cleavage and inactivation by serum proteases, e.g. trypsin (Labrou 2010).

In addition to serum proteases, it was reported quite recently by Patel and colleagues that two lysosomal cysteine proteases are able to degrade asparaginases in lymphoblasts, thus affecting the therapeutic effect of the L-asparaginases. Cathepsin B (CTSB), which is produced constitutively by normal and leukemic cells, was shown to be able to degrade both *E. coli* and *Erwinia* L-asparaginases, whereas asparaginyl endopeptidase (AEP) was able to use only *E. coli* L-asparaginase as its substrate. AEP is overexpressed principally in high-risk pre-B ALL patients and thereby destroys L-asparaginase activity and possibly triggers allergic reactions (Patel 2009).

One theory for asparaginase resistance is the secretion of asparagine by bone marrow mesenchymal cells, which offer a safe microenvironment for asparagine-dependent leukemic

cells (Iwamoto 2007). Resistance to asparaginase can also be caused by altered expression of genes involved in apoptosis (Holleman 2006) and protein synthesis (Holleman 2004).

2.3.6 Apoptosis and asparaginase

Apoptosis, also called programmed cell-death (PCD), is a highly regulated cascade of biochemical and morphological events involved in the elimination of damaged or harmful cells, and thus in the maintenance of cell homeostasis (Sprick 2004, Elmore 2007). Apoptosis is launched in response to a variety of either external or internal inducers, such as ultraviolet (UV) light, chemotherapeutic agents or DNA damage. According to current knowledge, the apoptotic cascade of events follows either the mitochondrial pathway (intrinsic route) or receptor-mediated (extrinsic) pathway.

During apoptosis, the apoptotic cells undergo typical morphological changes such as cell shrinkage and pyknosis, blebbing of plasma membrane, fragmentation of the cell nucleus, and finally the cells break into small membrane-surrounded particles, apoptotic bodies, which are phagocytosed without evoking any signs of inflammation (Elmore 2007). One of the molecular changes in apoptotic cells is the activation of caspases, cysteine aspartate specific proteases that cleave aspartate residues from numerous substrates during the early stages of apoptosis. Another hallmark is the movement of membrane lipid phosphatidylserine from the inner to the outer side of the plasma membrane thus serving as a recognition marker for phagocytes.

L-asparaginase-induced apoptosis was reported for the first time by Story and colleagues in 1993 when that group demonstrated DNA fragmentation and morphological changes in mouse lymphoma (LY-TH) cells after their exposure to L-asparaginase (Story 1993). Ueno and colleagues studied further the biochemical events leading to the death of murine L5178Y leukemic cells in the presence of L-aspraginase and they found that apoptosis is associated with the cell cycle arrest in G1 phase (Ueno 1997). Thereafter, asparaginase has been reported to induce apoptosis in natural killer cells (Ando 2005) and a subset of human sarcoma cells (Tardito 2007).

3 *Aims of the present study*

1. To study the effect of enzyme replacement therapy with recombinant human glycosylasparaginase in a mouse model of aspartylglycosaminuria.

2. To characterize the structure of a glycoasparagine present in the urine of an AGU patient as well as in the tissues of the AGU mouse model.

   To determine quantitatively the amount of glycoasparagine and GlcNac-Asn in AGU mice tissues as well as in AGU mice treated with enzyme replacement therapy.

3. To characterize the substrate specificities of two bacterial L-asparaginases, EcAII and ErAII, by utilizing β-aspartylpeptides as substrates

4. To evaluate the ability of recombinant human glycosylasparaginase to deplete the intra- and extracellular Asn reservoirs *in vitro* using EBV transformed lymphoblasts.

   To study the ability of GA to induce apoptosis in B- and T-lineage ALL cells and compare its cytotoxicity to clinically used bacterial L-asparaginases, EcAII and ErAII.

4 Materials and methods

4.1 REAGENTS

The reagents used in this study have been described in detail in the original papers (I-IV). β-Aspartylpeptides were prepared as previously described (Hanson 1964, Fastrez 1973) (III).

4.2 HUMAN RECOMBINANT GLYCOSYLASPARAGINASE (I, II, IV)

The recombinant human GA was stably overexpressed in NIH-3T3 mouse fibroblasts. The recombinant enzyme was purified with fast protein liquid chromatography (FPLC) by using gel filtration, DEAE-Sepharose and Mono-Q anion exchange columns as described previously by Kaartinen and colleagues (Kaartinen 1991). In the enzyme replacement therapy in the AGU mouse model, the enzyme was dialyzed into injection buffer containing 150 mM NaCl, 10 mM Tris-HCl and 1 mM β-glycerophosphate, pH 7,5 (I, II).

Dephosphorylation of recombinant GA (4.4 U) was carried out at 37 °C for 3.5 h in the presence of 1.6 U of alkaline phosphatase (ALP) in 50 mM Tris-HCl, pH 8, in a total volume of 1 ml. After incubation, the dephosphorylated GA was dialysed into phosphate buffered saline (PBS) and then sterile filtered. The final activity of the sterile dephosphorylated stock GA preparation was 6780 U/l.

4.3 CELL LINES

Mouse NIH-3T3 fibroblasts cultured in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% fetal calf serum (FCS) were used for the production of recombinant human GA (Mononen 1995) (I, II, IV).

Epstain Barr virus (EBV) -transformed GA-deficient lymphocytes were established as described (Volkman 1984) and cultured in RPMI-1640 supplemented with 15% FBS, 71 mg/ml streptomycin, 71 U/ml penicillin, and 2 mM L-glutamine. Those cells were used to study the ability of recombinant GA to deplete L-asparagine reservoirs in the cell culture medium as well as inside the cells (IV).

SUP-B15 cells established from the bone marrow of a 9-year-old boy with acute lymphoblastic leukemia (B cell precursor ALL) carrying the ALL-variant (m-bcr) of the BCR-ABL fusion gene (e1-a2) were bought from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and those cells were used in the cytotoxicity and apoptosis assays. Human T-lineage ALL CCRF-CEM cells established from the peripheral blood of a 3-year-old Caucasian girl with acute lymphoblastic leukemia (ALL) were from DSMZ (Braunschweig, Germany) (IV) and they were used in the cytotoxicity assays.

4.4 MOUSE MODEL OF ASPARTYLGLYCOSAMINURIA

The mouse model of AGU was generated as described elsewhere (Kaartinen 1996). The AGU mice in the enzyme replacement therapy experiments were aged from 9 months to 19 months (I, i.v. injections) or over one year (II, i.p. injections). In addition, 3- or 6-week-old AGU mice were

used to follow the effect of age on the accumulation of two glycoasparagines, GlcNAc-Asn and $Man_2GlcNAc_2$-Asn, in mouse tissues (II). In the analysis of GlcNac-Asn and $Man_2GlcNAc_2$-Asn in mouse tissues, the animals were anesthetized and then intracardially perfused with 0.9% NaCl (I, II). The collected tissues were stored at -70 °C until thawing and homogenization. Tissues were homogenized on ice in 50 mM Na-K-phosphate buffer, pH 7.5, containing 0.1 % Triton X-100 and then lysed by three freezing and thawing cycles and sonication.

4.5 ASSAY METHODS

4.5.1 Determination of L-Asn (IV), GlcNAc-Asn (I, II) and $Man_2GlcNAc_2$-Asn (II)

The concentrations of $Man_2GlcNAc_2$-Asn and GlcNAc-Asn as their phenylisothiocyanate (PITC) derivatives in tissues and urine, as well as the L-Asn level in the cell culture medium and inside the GA-deficient lymphoblasts were assayed by HPLC by using a Merck/Hitachi L-6200 liquid chromatograph (Hitachi Ltd., Tokyo, Japan) as described (Kaartinen 1990). CmCys was used as the internal standard.

The structure of $Man_2GlcNAc_2$-Asn in AGU mouse tissues was confirmed by sequential digestion by exoglycosidases α(1-6)-mannosidase, β-mannosidase and β-N-acetylglucosaminidase. The quantitative analysis of the degradation products as their 9-fluorenylmethyl chloroformate (FMOC) derivatives was performed by HPLC using CmCys as the internal standard (II).

4.5.2 Determination of GA activity (I, II, IV)

The activity of purified GA preparate (I, II, IV) and its activity in the cell lysates (IV) were determined by a fluorometric method (Mononen 1993) using β-(7-amido-4-methylcoumarin)-L-aspartic acid as a substrate. One unit of enzyme hydrolyzes 1 μmol of substrate per minute under standard conditions. GA activity in tissue homogenates was analyzed by HPLC as described (Kaartinen 1990) using CmCys as the internal standard.

4.5.3 Protein concentration

Protein concentrations of tissue homogenates, cell lysates and purified enzyme preparations were determined by using a Bio-Rad protein assay kit according to the manufacturer's instructions.

4.5.4 Enzyme kinetics of L-asparaginases using β-aspartylpeptides as substrates (III)

Determination of kinetic parameters of L-asparaginases by using β-aspartylpeptides as substrates was performed at 23 °C with a spectrophotometric assay (Tarentino 1969). Briefly, various substrate concentrations were incubated in microplate wells in the presence of NADH, MDH and ASAT for 15 minutes. Then, either *E. coli* L-asparaginase (Elspar®) or *Erwinia* L-asparaginase (Erwinase®) was added to achieve a final L-asparaginase activity of 91 U/l. Blank wells were incubated without enzyme and the blank values were subtracted from the actual measurements. The rate of the absorbance decrease at 340 nm was followed with a Tecan SpectraFLUOR spectrophotometer, Tecan Austria GmbH, Grödig, Austria, and the kinetic parameters were calculated using the Enzpack for Windows, version 1.4, Biosoft software package.

4.5.5 The cytotoxicity assay (IV)

The Cell Proliferation Kit I (MTT) Boehringer Mannheim GmbH (Mannheim, Germany) was used to determine the IC_{50} values for recombinant GA, dephosphorylated GA, *E. coli* L-asparaginase (Elspar®) and *Erwinia* L-asparaginase (Erwinase®) in CCRF-CEM and SUP-B15

leukemia cell culture. Shortly, various concentrations of the enzymes were incubated at 37 °C in microtiter plate wells in the presence of either SUP-B15 or CCRF-CEM cell suspension, and after three (SUP-B15) or four (CCRF-CEM) days of culture, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent was added into each well. After another 4 hours, the solubilization reagent was added to wells and the plate was incubated overnight at 37 °C. Finally, the absorbance of the wells at 540 nm was measured by using a Tecan SpectraFLUOR spectrophotometer, Tecan Austria GmbH (Grödig, Austria). ORIGIN™ Data Analysis Software, Microcal Software Inc., (MA, USA) was used to plot the dose-response curves.

4.5.6 Assay of apoptosis (IV)

In Situ Cell Death Detection Kit, TMR red (Boehringer Mannheim GmbH, Mannheim, Germany), which is based on the TdT-mediated dUTP nick-end labelling (TUNEL) method, was used to detect apoptosis both at the single cell level and in a cell population. SUP-B15 cells were incubated for two days in the presence of either recombinant GA, dephosphorylated GA (Elspar®) or *Erwinia* L-asparaginase (Erwinase®). After fixation and permeabilization, the cells were stained with tetramethylrhodamine (TMR) and 4',6-diamidino-2-phenylindole (DAPI), and the apoptotic (TMR, red) cells in the total cell population (DAPI, blue) were detected with a ZEISS Axioplan 2 imaging microscopy (Jena, Germany) and Isis in situ-imaging system software, MetaSystems GmbH (Altlussheim, Germany). Secondly, to determine the proportion of apoptotic cells in the whole cell population, the SUP-B15 cells were incubated in the presence of PBS (negative control), dephosphorylated GA or ErAII (positive control) for two days and after fixation and permeabilization the cells were stained with TMR. The cells were analyzed with a Coulter EPICS XL-MCL™ Flow Cytometer, Coulter Corporation (Miami, Florida, USA) and EXPO™ Analysis Software, Applied Cytometry Systems (Sheffield, UK).

5 Results

5.1 ENZYME REPLACEMENT THERAPY WITH RECOMBINANT HUMAN GLYCOSYLASPARAGINASE IN THE MOUSE MODEL OF AGU (I)

The level of GlcNAc-Asn accumulation was determined quantitatively in untreated AGU mice, wild type mice and in AGU mice treated with GA. It was shown that a single intravenous GA dose, 1 mg/kg, reduced the GlcNAc-Asn concentration by 94% in liver and 82% in spleen of the treated adult AGU mice. In heart, jejunum, kidney and heart the respective values were 16%, 54%, 17% and 29%. A single intravenous dose did not reduce the GlcNac-Asn concentration in brain tissue. An injection of 8 x 1 mg/kg of GA reduced the GlcNAc-Asn storage by 60-100% in the non-neuronal tissues of the treated AGU mice, but in brain tissue the accumulation remained unchanged. After eight injections of the GA at a dose of 10 mg/kg, GlcNAc-Asn was totally cleared from liver, spleen, kidney, heart and jejunum, and in brain tissue its accumulation was reduced by 20%.

After the GA treatment, the GA activity in the liver and spleen was dose-dependent – the higher the dose, the greater the GA activity. A single 1 mg/kg injection raised the activity in liver to 45% and in spleen to 21% of that of untreated wild-type mice, but in other tissues enzyme activity remained low or undetectable. After eight 1 mg/kg doses, the GA activity in liver and spleen was comparable to that of the untreated wild-type mice, but it remained unchanged in the other tissues. On the contrary, a marked increase in GA activity was detected in all non-neuronal tissues of adult AGU mice after the treatment protocol with 8 x 10 mg/kg. The highest GA activity was found in liver and spleen tissues, in which the activity was 87-fold and 67–fold higher than the corresponding value of untreated adult AGU mice. In the brain tissue, the activity of GA was 10% of that of wild-type mice.

The mean urinary excretion of GlcNAc-Asn of adult untreated AGU mice was 256 μmol/mmol creatinine (range 204-307 μmol/mmol creatinine). The ERT with either 1 mg/kg or 10 mg/kg intravenous dose every second day for 15 days reduced the excretion level of GlcNAc-Asn by 60% and 90% by the end of the therapy, respectively.

Examination of AGU mouse tissues by light and electron microscopy after the ERT with 8 x 10 mg/kg every second day revealed an effective correction of tissue pathology in the spleens and kidneys of treated AGU mice, whereas in their liver tissue, the vacuolization was not so clearly absent. Immunostaining with GA antibodies showed a wide distribution of the enzyme especially in the sinusoidal lining cells of the liver tissue as well as in the lymphoid cells and macrophages in the red pulp of the spleen of the treated AGU mice.

Plasma clearance of GA was found to be a two-phased process. The first rapid decline in serum GA activity, with a $t_{1/2}$ of 4 min, was followed by a slower phase with a half-life of 39 min. During the first phase, the enzyme had probably distributed within the vascular space or extracellular fluids, and during the slower phase it passed into tissues.

5.2 ACCUMULATION OF $MAN_2GLCNAC_2$-ASN IN AGU MOUSE TISSUES AND THE EFFECT OF ENZYME REPLACEMENT THERAPY WITH HUMAN RECOMBINANT GLYCOSYLASPARAGINASE (II)

5.2.1 Characterization of a glycoasparagine

In addition to GlcNac-Asn, AGU mouse tissues – especially liver - and urine showed massive accumulation of another compound by HPLC analysis. It was demonstrated by HPLC that the

compound was also not hydrolyzed by neuraminidase, α-fucosidase, β-galactosidase, β-mannosidase, β-N-acetylglucosaminidase nor *Erwinia* asparaginase (Erwinase®). On the contrary, the presence of α-mannosidase totally cleared the $Man_2GlcNAc_2$-Asn peak and the $ManGlcNAc_2$-Asn was formed. After the addition of β-mannosidase into the reaction mixture, $ManGlcNAc_2$-Asn was hydrolyzed to $GlcNAc_2$-Asn and finally after the incubation with β-N-acetylglucosaminidase only the GlcNAc-Asn peak was shown. It was demonstrated that the terminal mannose was not hydrolyzed by α(1-2)(1-3) mannosidase, but in the presence of α(1-6) mannosidase, it was rapidly cleared. In conclusion, the storage compound present in AGU mouse tissues and urine, as well as in AGU patient's urine, was characterized as a glycoasparagine carrying the tetrasaccharide moiety of α-D-Man-(1→6)-β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→N)-Asn ($Man_2GlcNAc_2$-Asn, Fig. 10).

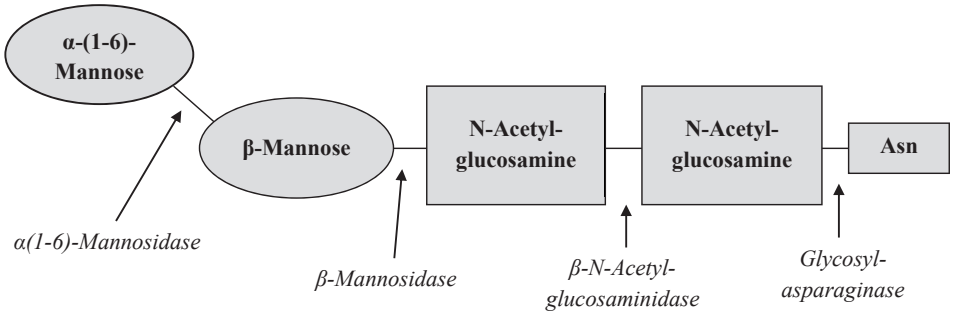


Figure 10. Structure of $Man_2GlcNAc_2$-Asn, and its hydrolysis by α(1-6)mannosidase, β-mannosidase, β-N-acetylglucosaminidase and glycosylasparaginase

5.2.2 Hydrolysis of $Man_2GlcNAc_2$-Asn by recombinant glycosylasparaginase

When an aliquot of AGU mouse liver tissue was incubated in the presence of GA both glycoasparagines, $Man_2GlcNAc_2$-Asn and GlcNAc-Asn, were hydrolyzed totally in 5 h (Fig 11). The reaction velocity of $Man_2GlcNAc_2$-Asn was 32,5 μM/min and that of GlcNac-Asn 34,4 μM/min.

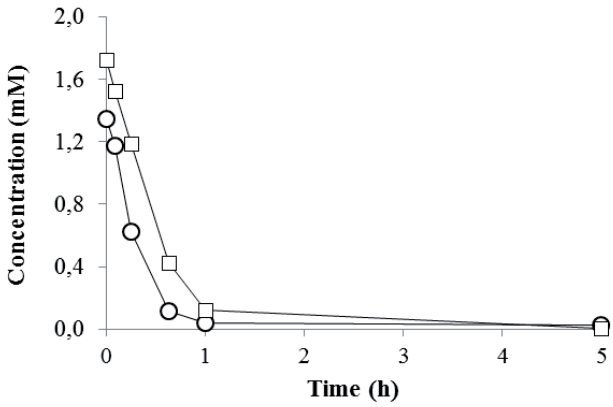


Figure 11. Hydrolysis of $Man_2GlcNAc_2$-Asn (○) and GlcNAc-Asn (□) in the presence of glycosylasparaginase (9.1 U/L) in AGU mouse liver extract

5.2.3 $Man_2GlcNAc_2$-Asn and GlcNAc-Asn in AGU mouse tissues

The most massive accumulation of $Man_2GlcNAc_2$-Asn was found in liver and spleen of adult AGU mice being 87% and 57% of the corresponding GlcNAc-Asn values. In other non-neuronal tissues of adult AGU mice, the level of $Man_2GlcNAc_2$-Asn was 28-76% of that of GlcNAc-Asn, whereas in brain, it was only one tenth of that of GlcNAc-Asn (Fig. 12). In liver and spleen, the concentration of $Man_2GlcNAc_2$-Asn increased with the age of the mice, whereas the GlcNAc-Asn accumulation in those tissues remained at the same level during the age period from 3 weeks to over one year. This type of age-dependence of the glycoasparagine storage was not found in other tissues.

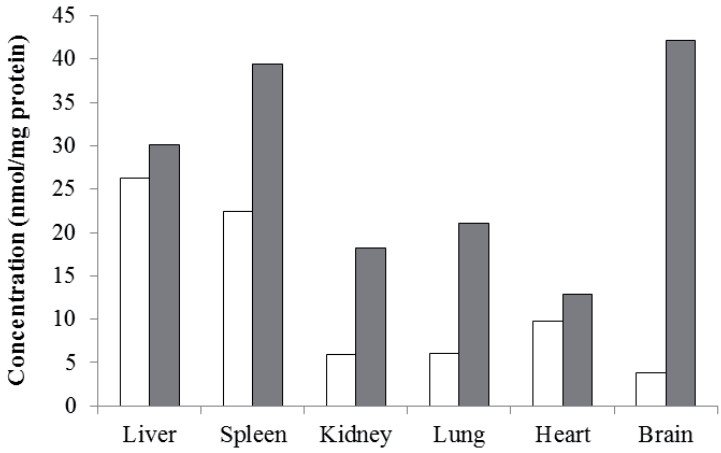


Figure 12. Accumulation of $Man_2GlcNAc_2$-Asn (white columns) and GlcNAc-Asn (grey columns) in adult AGU mouse tissues

5.2.4 Effect of ERT

After 13 intraperitoneal injections of GA, the activity of GA was found to be in the wild-type range in the liver of the treated AGU mice whereas in heart and lung enzyme activity was only 1% of that measured in the wild-type mice. The highest GA activity in the treated AGU mice was found in spleen, in which the activity was fourfold higher than in corresponding wild-type tissue. The therapy protocol effectively cleared the accumulation of both $Man_2GlcNAc_2$-Asn and GlcNAc-Asn from non-neuronal tissues of adult AGU mice by 55-98%, but less effectively, by 13-21%, from brain (see Table 8). Enzyme therapy with GA reduced the mean excretion of $Man_2GlcNAc_2$-Asn into the urine of adult AGU mouse by 72% and that of GlcNAc-Asn by 84%.

Table 8. Effect of ERT with GA on tissues of AGU mice

	[GlcNAc-Asn] % of KO	[MMGGA] % of KO	GA activity % of WT
Liver	2	3	84
Spleen	6	10	392
Kidney	29	21	0
Lung	33	34	2
Heart	45	8	1
Brain	79	87	0

5.3 HYDROLYSIS OF B-ASPARTYLPEPTIDES BY BACTERIAL L-ASPARAGINASES (III)

Two bacterial L-asparaginases derived from *E. coli* (Elspar®, EcAII) and *Erwinia chrysanthemi* (Erwinase®, ErAII) were capable of catalyzing the hydrolysis of several β-aspartylpeptides, particularly small size β-aspartyl amino acid amides. β-aspartylserineamide was hydrolyzed at half of the rate of L-Asn and β-aspartylalanamine or β-aspartylglycineamide had relative hydrolysis rates of 1/10-1/50 of that of Asn, respectively. The substrate specificity of the two bacterial L-asparaginases was rather similar with two exceptions: ErAII catalyzed the hydrolysis of H-Asp((Gly)$_4$-OH))-OH, but it was not hydrolyzed by EcAII, and secondly, EcAII could hydrolyze H-Asp(Phe-OMe)-OH, but this peptide remained unhydrolyzed by ErAII. These experiments were performed at 23 °C although in the human body at 37 °C the V_{max} may be even higher.

5.4 INDUCTION OF APOPTOSIS IN HUMAN T- AND B-LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA CELLS IN VITRO BY GLYCOSYLASPARAGINASE (IV)

Glycosylasparaginase was able to deplete both intra- and extracellular reservoirs of L-asparagine in the EBV-transformed GA-deficient lymphoblast culture. Hydrolysis of L-Asn from 2.3 to 0.2 mmol/l in the cell culture medium required a period of 4 hours in the presence of either 40 U/l GA or 10 U/l *Erwinia* L-asparaginase (Erwinase®). With lower GA activity, 10 U/l, the hydrolysis rate was remarkably slower and the Asn concentration of 0.2 mmol/l was not reached before 18 hours of incubation.

Intracellular Asn reservoirs of lymphoblasts were cleared in a dose-dependent manner – the higher the enzyme activity, the faster the hydrolysis. The highest Erwinase® activity, 1500 U/l cleared the Asn within one minute, but in the presence of enzyme doses 10 or 40 U/l of GA and 10 U/l of Erwinase®, the Asn concentration inside the cells initially increased during the first two hours of incubation and then gradually decreased to one tenth of the control cells within the next 6-18 hours. The enzyme activity inside the cells reached its peak in 1-2 hours and then gradually decreased within the next 24 hours.

GA induced cell death in two leukemic cell lines, human B cell precursor leukemia SUP-B15 cells and human T-lineage ALL CCRF-CEM cells, with similar efficacy than two bacterial L-asparaginases, Elspar® (EcAII) and Erwinase® (ErAII), which are routinely used in the treatment of ALL. The dephosphorylation of GA with alkaline phosphatase to prevent its transportation into cells did not affect its antileukemic efficacy; on the contrary, dephosphorylated GA was slightly more effective in killing the T-lineage ALL CCRF-CEM cells.

B-lineage ALL cell line SUP-B15 was 130-650 –fold more sensitive to all of the tested enzymes than T-lineage ALL CCRF-CEM cells, which was consistent with previous studies demonstrating the resistance of CCRF-CEM cells to L-asparaginase. The cytotoxicity of recombinant GA, dephosphorylated GA, ErAII and EcAII in B-lineage SUP-B15 ALL cell line was found to be rather similar. The IC_{50} value of each enzyme is listed in Table 9. The most effective enzyme in killing of SUP-B15 cells was EcAII which displayed an IC_{50} value of 4 U/l. The corresponding median IC_{50} values of ErAII, recombinant GA and dephosphorylated GA were 6 U/l, 12 U/l and 16 U/l, indicating that GA possesses significant cytotoxic activity of the same order of magnitude as the bacterial L-asparaginases used in ALL therapy. With an enzyme dose of 1000 U/l, ErAII killed 52% of the CCRF-CEM cells, whereas the corresponding dose of dephosphorylated GA, GA and EcAII killed 40%, 30% and 43% of the cells.

Table 9. The cytotoxicity of GA, dephosphorylated GA, ErAII and EcAII in SUP-B15 cells *in vitro*. IC_{50} value indicates that enzyme concentration required to induce death of half of the cell population.

Enzyme	**IC_{50} value (U/l)**
GA	12
Dephosphorylated GA	16
ErAII (Erwinase®)	6
EcAII (Elspar®)	4

Most of the SUP-B15 cells were apoptotic and displayed a red colour when incubated for two days at 37 °C in the presence of 100 U/l of dephosphorylated GA and stained with TMR and DAPI (Fig. 13). The proportion of apoptotic cells in the SUP-B15 cell population in the presence of either 100 U/l of dephosphorylated GA or 1,000,000 U/l ErAII was 65% and 69%, respectively.

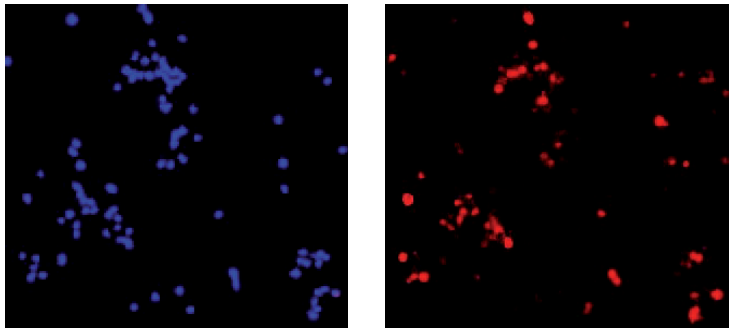


Figure 13. SUP-B15 cell population visualized under a fluorescence microscope with DAPI (blue, showing all the cells) and TMR (red, showing apoptotic cells)

6 Discussion

6.1 EFFECT OF ENZYME REPLACEMENT THERAPY IN AGU MOUSE MODEL

In recent decades, remarkable progress has been achieved in the enzyme replacement therapy of lysosomal storage diseases. However, the presence of BBB, which protects the CNS by selectively regulating the transport of molecules from the blood circulation into the CNS or vice versa (Fu 2011), complicates the development of treatment protocols for neurodegenerative LSDs. When this study was launched, enzyme therapy had been successful only in the non-neuronal tissues of LSD animal models and patients. Similarly, ERT with AGU had only been shown to be effective in cell cultures. As soon as the mouse model of AGU became available, it quickly proved to be a valuable tool for studying the effects of enzyme therapy on different tissues, also in brain.

In the first part of this work, intravenously administered recombinant GA at a high dose not only reached the non-neuronal tissues of the adult AGU mice, but also the brain. The increase in GA activity corresponding to 10% of the wild type activity led to a 20% decrease in GlcNAc-Asn concentration in the brain tissue. Furthermore, there was a complete clearance of GlcNAc-Asn from the liver, heart, kidney, jejunum, and spleen tissues. The results were better than expected in relation to the high age of the treated AGU mice.

The mechanism by which the high-dose enzyme is able to cross BBB of adult mice is still not known and the possible explanations are merely speculations. For example, it has been postulated that the enzyme may be taken up micropinocytosis or by other receptors than the MPRs (Blanz 2008), or there might be extracellular pathways that allow small amounts of large molecules to enter the CNS (Vogler 2005). One possible reason why some lysosomal enzymes can be delivered through the BBB whereas other large molecules are excluded may be due to the differences in the mode of uptake (Rozaklis 2011). GA is transported into AGU lymphoblasts and fibroblasts through mannose-6-phosphate mediated endocytosis (Dunder 2010) suggesting that there might be a specific M6P receptor responsible for GA delivery through BBB in AGU.

Recent studies with neurodegenerative LSDs have shown that it might be possible to overcome the BBB, at least to some extent. For example, ERT with the recombinant human α-mannosidase (rhLAMAN) in adult α-mannosidosis mice (Blanz 2008) was able to reach the CNS as high doses and successfully clear the accumulated substrate behind the BBB leading to a complete reversal of ataxic symptoms. However, the therapy did not significantly improve the neurocognitive and exploratory defects in these mice, probably due to the short duration of the experiment. Moreover, high-dose ERT proved to be effective in murine mucopolysaccharidosis VII brain using recombinant human β-glucuronidase (hGUS) (Vogler 2005).

In addition to the enzyme dose, the initiation of ERT at an early age is crucial for the successful therapy. For example, more recent ERT study with newborn AGU mice showed a twofold more effective clearance of the accumulated GlcNAc-Asn from the brain tissue than ERT in adult AGU mice (Dunder 2010). Interestingly, BBB of young mice was permeable to AGA even though the enzyme was administered intraperitoneally, although the i.p. route was not as effective as i.v. therapy. Access of enzyme through BBB of newborn mice was also studied with MPS IIIA by Gliddon and Hopwood, and according to their results, ERT with recombinant human sulfamidase is able to delay the development of behavior and learning difficulties in MPS IIIA mice if the therapy is started before the BBB maturation (Gliddon 2004).

ERT studies with other glycoproteinoses than AGU have shown variable results. For example, the treatment of sialidosis mice with recombinant neuraminidase (Neu1) evoked a significant reduction of lysosomal storage in systemic organs, but no improvement was detected in brain tissue. In addition, the treated mice developed a severe immune response towards the exogenous enzyme (Wang 2005). A similar trend was observed in the mouse model of galactosialidosis treated with Baculovirus-expressed protective protein/cathepsin A (PPCA) and neuraminidase (Neu1) (Bonten 2004).

Future alternatives to enable more significant access through BBB may benefit from the chemical modification of the therapeutic enzyme. For example, Grubb et al. tested the feasibility of ERT in MPS VII mice with sodium meta-periodate-oxidized β-glucuronidase (GUS); they demonstrated that the enzyme was successfully transported across the BBB thus correcting the storage in deficit neurons (Grubb 2010). In addition to its better uptake to brain tissue, the half-life of the modified enzyme in blood was remarkably prolonged. The modification of the enzymes reduces their clearance rate by liver and spleen and thus enables less frequent injections of the enzyme. Another ERT study with periodate-treated enzyme was performed by Rozaklis et al. by using chemically modified recombinant human sulfamidase (rhSGSH) in the MPS IIIA mouse model. It was found that periodate-treated enzyme could reach the brain tissue of MPS IIIA mice but it was unable to reduce the relative level of the accumulated substrates in brain cells. However, a modification of rhSGSH significantly improved the clearance of GlcNS-UA in non-neuronal tissues potentially due to its longer half-life in plasma (Rozaklis 2011).

It was demonstrated that the half life of the GA in serum was about 4 min in the first rapid phase followed by a half-life of 39 min during the slower phase. In liver tissue of AGU mice, the half-life of GA has been shown to be two days (Tollersrud 1988). There are potential techniques available to increase its half life; the enzyme could be covalently coupled to PEG or encapsulated inside red blood cells (Domenech 2011). Furthermore, the structure of GA could probably be tailored to achieve resistance to trypsin cleavage (Kotzia 2007) or to prevent the inactivation by lysosomal proteases Cathepsin B (Offman 2011) using similar techniques as used for bacterial asparaginases.

One interesting alternative to overcome the BBB of adults is to use epinephrine stimulation. Urayama and the colleagues found that transport of the enzyme P-GUS across the BBB could be induced in adult MPS VII mice by treating them with epinephrine and that this transport was attributable to the M6P receptor, the same receptor responsible for P-GUS transport in the neonate (Urayama 2007).

Before ERT can be made available for patients, even if the therapy seems to be effective in an animal model, much effort is needed to transfer the concept from the laboratory into the clinic. There exist many stages on the route from an idea to the ultimate approval and registration of the treatment. After the demonstration of clinical improvement in an animal model and availability of sufficient amounts of the therapeutic enzyme, scale-up of the therapeutic for human use as well as phase I, II and III clinical trials are needed (Hemsley 2011). The costs involved in this process are enormous and usually require funding by government and different societies and the participation of the pharmaceutical industry. It will be challenging to find a solution to the problem of developing cost-effective ERT. The currently used therapy protocols require frequent injections of expensive enzyme at relatively high-doses. Perhaps the chemical modification of the enzymes would translate into lower doses and less frequent injections of enzymes. There do seem to be ways to overcome the BBB since promising results have been achieved by direct injection of the therapeutic enzyme into cerebrospinal fluid. This therapeutic approach is currently in clinical trials with MPSI and MPS IIIA, yet it remains to be clarified which dosage schedule and injection route will provide the best results. In addition, gene therapy is a promising approach for the treatment of LSDs, because currently there are virus vectors available able to cross the BBB, as confirmed with MPS IIIB mice (Hawkins-

Salsbury 2011). Combination therapy may be more effective than a single therapy. However, every LSD is different and thus the optimal combination has to be found for each disease.

At present, there are no clinical trials investigating the efficacy of ERT ongoing for neurodegenerative LSDs but human trials are planned for α-mannosidosis. The therapy of every LSD should be started as soon as possible before irreversible damage has occurred. Thus, newborn screening is important in order to make the correct diagnosis as early as possible.

In the light of the present results and the most recent ERT studies with AGU mice, enzyme replacement therapy with GA may be beneficial and the therapy should be started as soon as possible before the newborn's BBB is fully mature. In addition, the optimization of the GA dosage and the frequency of the administrations may reduce the storage of GlcNAc-Asn more effectively in brain tissue, thus improving also the cognitive impairment. The promising results with the modification of therapeutic enzymes suggest that also the half-life of GA may be prolonged.

6.2 GLYCOPROTEIN $MAN_2GLCNAC_2$-ASN IN AGU MOUSE TISSUES

In this study, a closer look was taken to a glycoprotein present in AGU mouse tissues and urine. The structure of the glycoprotein was confirmed as $Man_2GlcNAc_2$-Asn (MMGGA) and it was found at concentrations as high as 30–87% of those of GlcNac-Asn, in non-neuronal tissues of untreated AGU mice. MMGGA fragments have also been found in the liver, spleen, kidney, thyroid and brain of AGU patients (Gordon 1998, Maury 1980) albeit their quantitative amounts in tissues have not been studied. One possible reason for the accumulation of MMGGA is that the α-(1-6)-linked mannose of the pentasaccharide core unit in Asn-linked glycoproteins cannot be hydrolyzed by α(1-6) mannosidase before removal of Asn by GlcNac-Asn, as demonstrated by Haeuw (Haeuw 1994).

The accumulation of MMGGA in the liver and spleen of the untreated AGU mice increased with the age of the mice. The probable reason for the age-dependent storage may be disturbed function of chitobiase in the presence of high amounts of GlcNAc-Asn and MMGGA in the lysosomal milieu. In contrast to GlcNAc-Asn, the amount of MMGGA in brain tissue was insignificant, only one tenth of GlcNAc-Asn, suggesting that it has only a minor role in the central neuronal system symptoms encountered in AGU. The reason for the low MMGGA concentration in brain tissue might be due to the ability of the major lysosomal mannosidase to hydrolyze α(1-6)-mannose (Aronson 1999). In addition, it has been suggested that chitobiase activity is higher in brain than in liver (Fisher 1992). Furthermore, there might be alternative degradation pathways present in brain.

ERT delivered via 13 intraperitoneal injections of recombinant GA effectively cleared MMGGA as well as GlcNAc-Asn from the non-neuronal tissues in amounts ranging from 55% to 98%. The highest GA activity was found in liver and spleen of the treated AGU mice whereas in heart and lung, the activity was negligible. It has been also demonstrated with other LSD animal models that there is variation in the uptake of the enzymes into different tissues. These differences have been attributed to the enzyme itself as well as its level of phophorylation and glycosylation (Roces 2004). The degradation rate of MMGGA in liver tissue extract of an AGU mouse was 94% of that of GlcNac-Asn. This result is in line with the kinetic spectrophotometric analysis conducted by Kaartinen et al. (Kaartinen 1992) which calculated a relative rate 85% of that of GlcNAc-Asn for high mannose type glycoasparagine $Man_6GlcNac_2$-Asn. After receiving 13 i.p. injections of recombinant GA, the excretion of MMGGA into urine of AGU mice was reduced by 72% and that of GlcNac-Asn by 84%. The urinary excretion of MMGGA and GlcNAc-Asn was not corrected to the normal level even with the highest enzyme dosage, indicating that these compounds continue to leak into the systemic circulation from the tissues that are not accessible to the glycosylasparaginase.

The accumulation of GlcNAc-Asn in AGU patients' tissues and body fluids is considered to be the main reason for clinical symptoms of the disease, but the present results suggest that the massive accumulation of large glycoproteins, including MMGGA, may well have also a significant role in the progressive pathogenesis of AGU.

6.3 ENZYMATIC PROPERTIES OF HUMAN RECOMBINANT GLYCOSYLASPARAGINASE AND BACTERIAL L-ASPARAGINASES

Glycosylasparaginase and L-asparaginase share both structural and biochemical properties, such as a polymeric subunit structure and the ability to hydrolyze L-asparagine to L-aspartic acid. Bacterial *E. coli* and *Erwinia chrysanthemi* L-asparaginases were capable of catalyzing especially the hydrolysis of small size β-aspartyl amino acid amides β-aspartylserineamide, β-aspartylalanamine and β-aspartylglycineamide. Similarly to GA, bacterial asparaginases can hydrolyze β-aspartylserineamide at a relatively higher rate than they can break down other β-aspartylpeptides. All of the tested β-aspartylpeptides were shown to be hydrolyzed by GA, but three of them were not hydrolyzed at all by the asparaginases. In addition, the relative hydrolysis rates of H-Asp(Gly-OH)-OH, H-Asp(Phe-OMe)-OH and H-Asp((Gly)$_4$-OH))-OH were negligible when compared to those of GA (see Table 10).

Table 10. Relative hydrolysis rates of *E. coli* asparaginase (EcAII), *Erwinia* asparaginase (ErAII) and glycosylasparaginase (GA) against β-aspartylpeptides

	Relative rate (%)		
Compound	**EcAII**	**ErAII**	**GA[a]**
H-Asn-OH	100	100	41
H-Asp(Ser-NH_2)-OH	52	53	71
H-Asp(Ala-NH_2)-OH	8,2	8,6	35
H-Asp(Gly-NH_2)-OH	2,2	2,1	15
H-Asp(Gly-OH)-OH	0,3	0,1	3
H-Asp(Phe-OMe)-OH	0,2	0	23
H-Asp((Gly)$_4$-OH))-OH	0	0,3	8
H-Asp(Gly-Gln-OH)-OH	0	0	27
H-Asp(Gly-Val-OH)-OH	0	0	49
H-Asp(GlcNAcNH)-OH	0	0	100

[a]Relative rates for GA have been determined by Noronkoski et al. 1998.

The reason why GA is able to hydrolyze a wider range of β-aspartylpeptides than L-asparaginase is most probably due to the difference in the binding site. GA has more a spacious active site, placing the L-asparagine part of the substrate into a narrow pocket and allowing variable-sized parts, such as the oligosaccharides, to extend outwards (Wang 2007). Bacterial asparaginases will not accommodate substrates significantly larger than glutamine as active site ligands (Aghaiypour 2001). In the plant-type asparaginases, access of too long molecules, such as L-glutamine, is prevented by the arginine side chain, which controls the length and exact positioning of a substrate in the active site (Michalska 2006).

The present results suggest that the high doses of L-asparaginase used in the treatment of ALL could hydrolyze the β-aspartylpeptides present in human body thus causing some of the side effects.

6.4 ANTILEUKEMIC CHARACTERISTICS OF GA *IN VITRO*

The glutaminase activity of clinically used bacterial L-asparaginases has been linked to their therapeutic toxicity. Glycosylasparaginase, which is able to hydrolyse asparaginase without affecting glutamine metabolism, was tested in this study as a potential antileukemic drug *in vitro*. At first, GA was shown to deplete both the intra- and extracellular Asn reservoirs *in vitro* using EBV transformed GA-deficient lymphoblasts. Thus, GA fulfills the demand of an antileukemic drug to minimize especially the extracellular L-Asn concentration. As a result, those leukemic cells which lack asparagine synthase and require an exogenous source of asparagine for protein synthesis cannot survive.

Furthermore, it was demonstrated that dephosphorylated GA shows similar cytotoxicity against the B-lineage leukemic cell line SUP-B15 when compared to bacterial asparaginases and native phosphorylated GA. In addition, a dose-dependent growth inhibition could be demonstrated in human T cell leukemia CCRF-CEM cells *in vitro*, but it was less effective in the presence of dephosphorylated GA or phosphorylated GA than the bacterial asparaginases. Finally, both dephosphorylated and phosphorylated GA were shown to induce the key feature of apoptosis, DNA fragmentation, in SUP-B15 cells.

In conclusion, due to the comparable antileukemic characteristics of dephosphorylated GA, phosphorylated GA and bacterial L-asparaginases *in vitro*, GA might have potential as an anticancer-drug. In addition, GA as an endogenous human protein, might cause less side effects than the bacterial asparaginases. It is recommended that a further investigation using human leukemia xenograft mouse models could help clarify the antileukemic efficacy, pharmacokinetics and pharmacodynamics of glycosylasparaginase therapy *in vivo.*

7 Conclusions

The aim of this thesis was to investigate the effects of enzyme replacement therapy with human recombinant glycosylasparaginase on the mouse model of AGU. A further goal was to study the substrate specificity of bacterial L-asparaginases by using β-aspartylpeptides as substrates. The final goal was to evaluate the antileukaemic properties of recombinant glycosylasparaginase by studying its cytotoxicity in comparison with two bacterial L-asparaginases in two ALL cell lines *in vitro*. The following conclusions of the thesis work can be drawn:

1. This is the first time that enzyme replacement therapy with human recombinant glycosylasparaginase was tested in a mouse model of AGU. It was found that GA effectively corrected the GlcNAc-Asn storage in non-neuronal tissues of adult AGU mice, and its activity reached also the brain tissue. The disappearance of intravenously administered GA from the systemic circulation was found to be two-phased, the first phase having a $t_{1/2}$ of 4 min followed by a slower phase with a $t_{1/2}$ of 39 min.

2. The structure of a glycoasparagine, $Man_2GlcNAc_2$-Asn, was characterized and its massive accumulation was quantitatively determined in the non-neuronal tissues and the urine of adult AGU mice. In contrast to GlcNAc-Asn, the concentration of $Man_2GlcNAc_2$-Asn in brain tissue was very low. The accumulation of $Man_2GlcNAc_2$-Asn in the liver and spleen of AGU mice was found to be age-dependent, being higher in older mice. Enzyme replacement therapy administered as i.p. injections of GA, was successful in reducing the accumulation of $Man_2GlcNAc_2$-Asn and GlcNAc-Asn in non-neuronal tissues and urine of the AGU mice, but less effective in brain.

3. Two bacterial L-asparaginases were shown to hydrolyze β-aspartylpeptides, some of which are present in the human body. Both L-asparaginases catalyzed most effectively the hydrolysis of β-aspartylpeptide amides; β-aspartylserineamide was hydrolyzed by EcAII and ErAII at half of the rate of L-Asn, and the hydrolysis rates of β-aspartylalanineamide and β-aspartylglycineamide were 1/10-1/50 of that of Asn.

4. Human recombinant glycosylasparaginase was demonstrated to possess antileukaemic properties. GA was able to hydrolyze L-asparagine to L-aspartic acid *in vitro* in cell culture medium and inside the cultured EBV-transformed GA-deficient lymphoblasts. Secondly, GA was able to induce cell death, especially in B-lineage ALL cells, and also in T-lineage ALL cells *in vitro* in a manner comparable with the two bacterial L-asparaginases used in ALL therapy.

8 References

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Catalytic and Therapeutic Characteristics of Human Recombinant Glycosylasparaginase and Bacterial L-asparaginases

Aspartylglycosaminuria (AGU), an inherited lysosomal storage disease, is caused by the deficient activity of a lysosomal enzyme glycosylasparaginase (GA).
Loss of GA activity results in the accumulation of glycoasparagines in tissues leading to progressive psychomotor retardation and a shortened life span. Currently there is no cure for AGU. In this study, the effectiveness of enzyme replacement therapy (ERT) with human recombinant GA was evaluated in the mouse model of AGU. Furthermore, the enzymatic properties of GA and bacterial asparaginases were compared. This study reveals new therapeutic and catalytic properties of human GA and bacterial asparaginases.

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